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THE SULFHYDRYL GROUPS OF PHOSPHORYLASE

by

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A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "THE SULFHYDRYL GROUPS OF PHOSPHORYLASE", submitted by Mary Louise Battell in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The sulfhydryl groups of glycogen phosphorylase from rabbit muscle have been investigated with respect to their reactivity with PCMB, DTNB, cystine, NEM, iodoacetic acid and iodoacetamide. By spectrophotometric titration, PCMB reacts with 6 of the 18 half-cystine residues in native phosphorylase b, and with 12 - 14 of the 36 half-cystine residues in native phosphorylase a, and causes complete inactivation in both cases. Reaction of approximately 6 more residues in phosphorylase b (12 in phosphorylase a) occurs when the protein is denatured with HCl or SDS.

DTNB, cystine and iodoacetic acid react with only two sulfhydryl groups in native phosphorylase b, and do not cause any loss of activity. Iodoacetamide can also react with two sulfhydryl groups without causing any loss of enzymic activity, but activity is lost in proportion to the extent of titration of the next four groups. DTNB reacts with 12 groups in SDS denatured phosphorylase b but with only 9 groups in urea or guanidine HCl denatured enzyme. Cystine reacts with fewer groups than DTNB in denatured protein regardless of the denaturing agent chosen. NEM and iodoacetic acid react with 14 groups in urea denatured phosphorylase b. Two experiments are reported that provide additional confirmation for previous reports of the absence of disulfide bridges in phosphorylase.

We conclude that two sulfhydryl groups per mole of phosphorylase b can react with any reagent without loss of enzymic activity at a reaction rate that is of the same order of magnitude as that of the -SH group of glutathione. When the correct reagent and conditions are chosen, a further class of 4 sulfhydryl groups reacts, resulting in the complete loss of enzymic activity. Thus, only two specific sulfhydryl groups on each monomer unit need be titrated in order to inactivate glycogen phosphorylase and cause it to dissociate.

The two peptides containing these two sulfhydryl groups have been labelled with ^{14}C iodoacetamide, isolated from a pepsin digest of the protein, and identified with two of the cysteine containing peptides previously sequenced by other members of this department. These two peptides are ala-cys-ala-phe (N) and asn-ala-cys-asp (A).

The rate constants for the reaction of ^{14}C-iodoacetamide with each of three individual sulfhydryl groups for both phosphorylase a and b have been measured and compared to the overall rates of inactivation. Phosphorylase a reacts about four times as fast as does phosphorylase b. There is also a difference between the two enzyme forms with respect to the relationship of the two particular sulfhydryl groups (N and A) to activity. A mechanism has been proposed to account for their differences.

Upon denaturation of phosphorylase by several of the usual protein denaturants, another class of sulfhydryl groups reacts readily, the exact number depending on the reagent and the conditions, but the complete titration of all 18 sulfhydryl

groups per molecule of phosphorylase b occurs only under special conditions. These results are of general relevance to procedures employing various protein denaturants and sulfhydryl reagents for the titration of protein sulfhydryl groups.

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LIST OF ABBREVIATIONS

AMP	-	adenosine 5'-monophosphate
cpm	-	counts per minute
dpm	-	disintegrations per minute
DTNB	-	5,5'-dithiobis(2-nitrobenzoic acid)
EDTA	-	ethylene diamine tetraacetic acid disodium salt
IAA	-	iodoacetic acid
IAM	-	iodoacetamide
NEM	-	N-ethylmaleimide
PCMB	-	p-chloromercuribenzoic acid
SDS	-	sodium dodecylsulfate
-SH	-	sulfhydryl
TCA	-	trichloroacetic acid

INTRODUCTION

Reaction of the -SH groups of glycogen phosphorylase causes a loss of activity and structure of the enzyme isolated from rabbit skeletal muscle. Thus, the enzyme, which is responsible for the breakdown of glycogen *in vivo*, joins many other enzymes which are classed as sulfhydryl enzymes because -SH groups which they possess are, in some way, related to their activity. Despite extensive studies of "sulfhydryl enzymes", and the growing number of specific reagents, very little is known about the relationship of -SH groups to activity in enzymes.

The -SH groups of proteins are of considerable chemical interest since they are the most highly reactive of the amino acid side chains. The various types of reactions which they can undergo, including oxidation, mercaptide formation, alkylation, and addition to double bonds, have been reviewed most recently by Webb (1), and previously be Cecil (2), Johnstone (3), Madsen (4), and Benesch and Benesch (5). The additional factor of varying reactivity of -SH groups as they occur in proteins for, as yet, unexplained reasons, makes their study doubly interesting.

The molecular weight of phosphorylase *a* was first established by Keller and Cori (6) as 495,000 in 1953. The corresponding molecular weight for phosphorylase *b* is 242,000 (see Brown and Cori, 7). Recent work by Seery *et al.* (8) has

established that the molecular weights are 370,000 and 185,000, respectively, while the monomer is 92,500. These values have been independently confirmed by DeVincenzi and Hedrick (9). The sedimentation constants reported by Keller and Cori (6) and Madsen and Cori (10) were correct (see 11). They were 12.2 S, 8.2 S, and 5.6 S for the tetramer, dimer, and monomer, respectively. All values in this paper which depend on molecular weight are corrected to the new molecular weights. Phosphorylase b is transformed into phosphorylase a by the action of phosphorylase kinase which phosphorylates a particular serine residue concomitantly with a dimerization reaction. The phosphorylated serine residue is perhaps the only physical feature which clearly distinguishes phosphorylase a from phosphorylase b, since it was established in 1965 that phosphorylase a can exist as a dimer under normal conditions of activity measurement (11, 12, 13).

In 1956, when Madsen and Cori (10, 14, 15) studied the -SH groups of phosphorylase a, they found that titration of 18 -SH groups per mole of enzyme led to loss of 100% of the enzyme activity. (Since the molecular weight of the tetramer was then thought to be 495,000, there were 18 -SH titratable at neutral pH per 500,000 gm of protein, or 13 -SH groups per mole according to the new molecular weight.) The reaction also caused complete conversion to monomer. Since titration of the protein with varying amounts of PCMB indicated that each of the 18 -SH groups had a similar relationship to

activity, it was assumed that the reaction of the -SH groups was causing a loss of structure, and that the -SH groups were not directly concerned with the activity of the enzyme; in other words, there were no -SH groups at the active center or centers.

In 1963, Appleman *et al*. (16) determined the total half-cystine content of phosphorylase *b* by performic acid oxidation of the enzyme and measurement of the cysteic acid content on the amino acid analyzer. They obtained 22 moles per mole of phosphorylase *b* or 44 moles per mole of phosphorylase *a* based on the molecular weights 242,000 and 495,000, respectively. There is an obvious discrepancy here between the value, 18, obtained by Madsen and Cori by the PCMB titration and the 44 moles of cysteic acid obtained by Appleman *et al*. Appleman also treated the protein with iodoacetic acid in the presence of SDS and sodium borohydride. They found 18.2 moles of carboxymethylcysteine plus half-cystine plus cysteic acid (17). This value confirmed that there were certainly more half-cystine residues than those revealed by the PCMB titration but left some doubt about the total number of groups.

This work was intended to determine the total number of -SH groups and disulfides in the various forms of phosphorylase, to examine their reactivity toward the wide variety of specific -SH reagents now available, and to begin the study of their role in the biological activity and the quaternary structure of the enzyme.

MATERIALS AND METHODS

Crystalline rabbit muscle phosphorylase b was prepared as described by Fischer and Krebs (18) and crystalline phosphorylase a was prepared by the method of Green and Cori (19) except that mercaptoethanol replaced cysteine in both procedures. The enzymes were recrystallized at least three times. Immediately before use, phosphorylase b was passed through a Sephadex G-25 gel filtration column pre-equilibriated with the appropriate buffer. Phosphorylase a was washed several times with cold buffer.

Rabbit liver glycogen, purchased from Sigma Chemical Co., was purified by passage through a Dowex 1-Cl column. Glucose-1-phosphate, AMP, PCMB (free acid), and iodoacetamide were also purchased from Sigma Chemical Co. Iodoacetic acid was obtained from Eastman Organic Chemicals; DTNB from Calbiochem. Iodoacetic acid-2-^{14}C (7 mc/mM), iodoacetamide-1-^{14}C (5 mc/mM), and D,L-cystine-3-^{14}C (3 mc/mM) were purchased from the Radiochemical Center, Amersham. ^{14}C-iodoacetic acid was dissolved in hot benzene and crystallized with an excess of non-radioactive material which had previously been recrystallized from benzene-petroleum ether; the radioactive iodoacetamide was similarly recrystallized from hot water. N-ethyl-1-^{14}C maleimide (1.25 mc/mM) was obtained from New England Nuclear; PCMB-1-^{14}C (10 mc/mM) from Calbiochem. Mann Research Laboratories, Ltd. Ultra Pure urea was used and the sodium dodecyl sulfate was obtained from Sigma.

Phosphorylase activity was measured in the direction of glycogen synthesis according to Cori *et al.* (20), using 0.02 M sodium β-glycerophosphate, 1.5 mM EDTA, pH 6.8 as the buffer to dilute the enzyme to the appropriate protein concentration. Mercaptoethanol was included in this buffer to a final concentration of 0.05 M when necessary to stop the iodoacetamide reaction. The substrate for the reaction was 0.032 M glucose-1-phosphate, 2% glycogen, and 2 mM AMP. Reactions with phosphorylase *a* were done in the presence and absence of AMP.

Protein concentration was determined from the absorbance at 280 mμ, using a Zeiss PMQ II spectrophotometer and using a value for $E_{cm}^{1\%}$ of 13.2 (21). The method of Lowry *et al.* (22) was used whenever reagents interfered with the spectrophotometric determination.

The PCMB titration of Boyer (23) was carried out as follows. Crystals of phosphorylase *a* were washed with cold 0.02 M glycerophosphate buffer, pH 6.8, and dissolved in 1% KCl (pH about 7); then sufficient 1 N HCl was added to lower the pH to 2.3. The acid was added rapidly to prevent precipitation of the protein at intermediate pH's. Various amounts of the protein were added to cuvettes which contained an aliquot of 0.1 M glycine buffer to stabilize the pH and sufficient water to bring the volume to the same final value. Finally, PCMB was added to each cuvette, and the increase in absorbance at 255 mμ determined against a cuvette which

contained the same concentration of PCMB but no protein. Absorbance was read until it reached a constant value. The absorbance due to the protein was subtracted, and the increase in absorbance due to formation of mercaptide plotted against protein concentration. The concentration of PCMB was determined from the absorbance at 234 mμ under the same conditions (0.1 M glycine buffer, pH 2.3) using the determined extinction coefficient of 17,400. Phosphorylase b was titrated by the same method except that the protein was initially passed through a gel filtration column in 1% KCl.

In the experiments in which the protein was denatured with SDS (final concentration, 1%), 0.02 M sodium glycerophosphate, pH 6.8, was used throughout the procedure, since KCl causes precipitation of dodecyl sulfate. Measurements of mercaptide formation were made at 250 mμ. PCMB concentration was determined at 232 mμ using the extinction coefficient of 18,000 which was determined in 0.02 M sodium glycerophosphate buffer, pH 6.8.

The chelating agent, EDTA, was not used in any step in the spectrophotometric titration with PCMB because Boyer (23) established that it interfered with the observation of the reaction.

Spectrophotometric titrations of phosphorylase were also done using DTNB according to Ellman (24) except that 0.1 M sodium glycerophosphate, 1 mM EDTA, pH 6.8, was the buffer used. The reagent was added to cuvettes containing

a measured amount of protein, and the absorbance at 412 mμ measured. The extinction coefficient, 13,600 was used to calculate the number of moles of -SH groups reacting. When SDS was used, it was made up as a concentrated solution in glycerophosphate buffer, and added to the protein (in amounts to give a final concentration of 1%) 20 - 30 minutes before the addition of the DTNB. Experiments were also performed on protein solutions made 8 M in urea and 5.2 M in guanidine HCl (final concentrations).

The total half-cystine content of phosphorylase was determined by performic acid oxidation (25) of the enzyme for 4 hours at 0°. Samples of phosphorylase were dialyzed against either deionized water or against 0.1 mM EDTA before the oxidation. After hydrolysis, the samples were assayed on a Beckman Spinco amino acid analyzer. The number of moles of each amino acid was determined in comparison with the value for alanine obtained by Appleman et al. (16, 17). Unless otherwise stated, protein hydrolysis was carried out for 20 to 24 hours at 110° in redistilled 6 N HCl.

The stability of carboxymethylcysteine with time of hydrolysis was investigated. Protein that had been reacted with iodoacetic acid for 12 hours in 8 M urea was adjusted to pH 3 and then dialyzed against 0.001 N HCl to remove excess reagent. Samples of freeze-dried material were hydrolyzed in 6 N HCl for 12 hours, 24 hours, 48 hours, and 72 hours. Carboxymethylcysteine was not completely liberated

in the 12 hours sample, but gave the same value from 24 hours to 72 hours. A similar result was obtained by Noltmann et al. (26).

Incorporation of various radioactive reagents was determined by pipetting aliquots of reaction mixture into filter funnels containing 10 ml of cold 5% trichloroacetic acid. Either Gelman triacetate or Millipore filters placed in the bottom of the funnel were used to collect the precipitated protein. After the precipitate was washed four times with 5 ml aliquots of cold 5% TCA, the filter was sucked dry and transferred to a scintillation vial. Ten ml of Bray's scintillation fluid (27) without ethylene glycol was added to the vials and the samples counted to an error of 1% in either a Nuclear-Chicago Mark I or a Beckman LS-200B or CPM-100 liquid scintillation counter. The cpm were corrected to dpm using a quench correction curve determined for the system being used. The amount of radioactivity incorporated into the protein was then compared to the total radioactivity in the reaction mixture.

It was found that protein which had been treated with SDS could not be precipitated with cold 5% TCA whereas protein denatured with urea, guanidine HCl, or acid precipitated readily. 10% TCA gave a very fine precipitate of SDS denatured protein which tended to clog the filter and prevent subsequent washing. Two concentrations of perchloric acid (7% and 15%) gave approximately the same results. However,

protein denatured with SDS could be pipetted onto a filter paper disc (2.3 cm, Whatman), dried in a stream of warm air, and subsequently washed with first cold 10% TCA, then with cold 5% TCA. A Lowry test indicated that no protein was washed off the paper disc. The method described was used in experiments where incorporation into SDS-denatured protein was measured.

High-voltage electrophoresis was carried out on Whatman papers, either #1 or #3 MM, using 0.6 mg protein per cm in the former case and 2.5 mg per cm in the latter case. The buffers used were as follows:

pH 6.5	879 ml deionized water	100 ml pyridine	3 ml glacial acetic acid

Coolant: 92% toluene, 8% pyridine by volume

pH 3.5	1890 ml deionized water	10 ml pyridine	100 ml glacial acetic acid

Coolant: Varsol

pH 1.8	2% formic acid	8% acetic acid

Coolant: Varsol

Amino terminal residues were determined by reaction with 1-dimethyl aminonaphthalene-5-sulfonyl chloride (28, 29). Sequential degradation was carried out according to the procedures of Edman (30, 31, 32).

The Beckman Spinco Model E analytical ultracentrifuge was used for determination of sedimentation coefficients. A speed of 59,780 rpm was used at a temperature of 20°. The

centerpiece was Kel-F. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components.

Side strips of electropherograms were analyzed for radioactivity using a Nuclear-Chicago Actigraph III strip scanner with 4 π geometry. Analysis was done using a slow scan speed (15 cm/hr) and a long time constant (50 secs).

EXPERIMENTAL RESULTS

I. Cysteic Acid Determination

Duplicate samples of phosphorylase *a* gave 18.0 ± 0.4 moles of cysteic acid per 185,000 gm of protein, while duplicate samples from two different experiments on phosphorylase *b* gave values (averaged) of 18.1 and 16.6 moles per 185,000 gm of protein. The average of these results is 17.6 moles per 185,000 gm of protein. This value (which is just slightly higher than that reported by Appleman *et al.* (16) yields 9 as the closest integral number of moles per phosphorylase monomer, and 18 moles per mole of phosphorylase *b* or 36 moles per mole of phosphorylase *a*. The values stated are corrected for 6% hydrolytic loss (25).

II. PCMB

Fig. 1 shows a typical titration of phosphorylase by PCMB, in which a constant amount of PCMB was added to cuvettes containing varying amounts of protein which had been denatured with acid. The results of the work with PCMB are compiled in Table I. It is evident that the same number of -SH groups react (per dimer) regardless of whether phosphorylase a or b is used. Further, as is often the case with reaction of protein -SH groups (2), denaturation of the protein leads to reaction of additional -SH groups. However, even in the presence of 1% SDS, all of the half-cystine residues do not react with PCMB.

A complete titration of the -SH groups was possible when the protein was first digested with pepsin. At this pH, disulfide interchange does not take place (33, 34), and autoxidation is limited. After digestion, a titration was done immediately upon bringing the pH of the reaction mix to 7.2. The result is shown in Fig. 2 where 16.2 groups per mole of phosphorylase b reacted with PCMB. A second experiment gave 18.5 -SH per mole. This is good evidence that there are no disulfide bridges in phosphorylase.

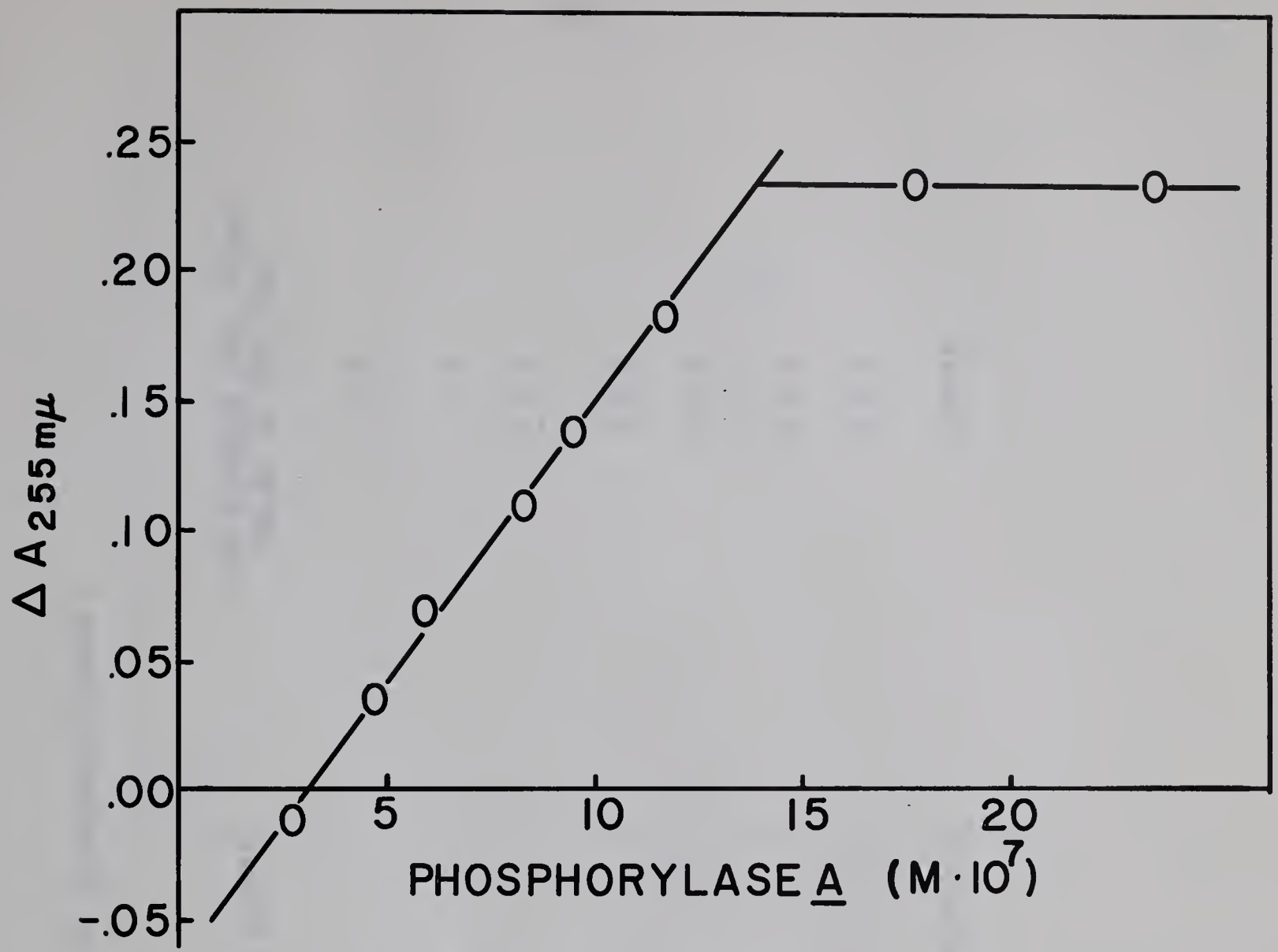


Figure 1: Spectrophotometric titration of the -SH groups of phosphorylase a by PCMB at pH 2.3 in 0.1 M glycine buffer. 3.75×10^{-5} M PCMB was added to cuvettes containing the concentrations of phosphorylase a in glycine buffer shown on the figure. The break in the line occurs at 13.9×10^{-7} M phosphorylase a, that is, at the point where 27 -SH groups per mole of enzyme have reacted.

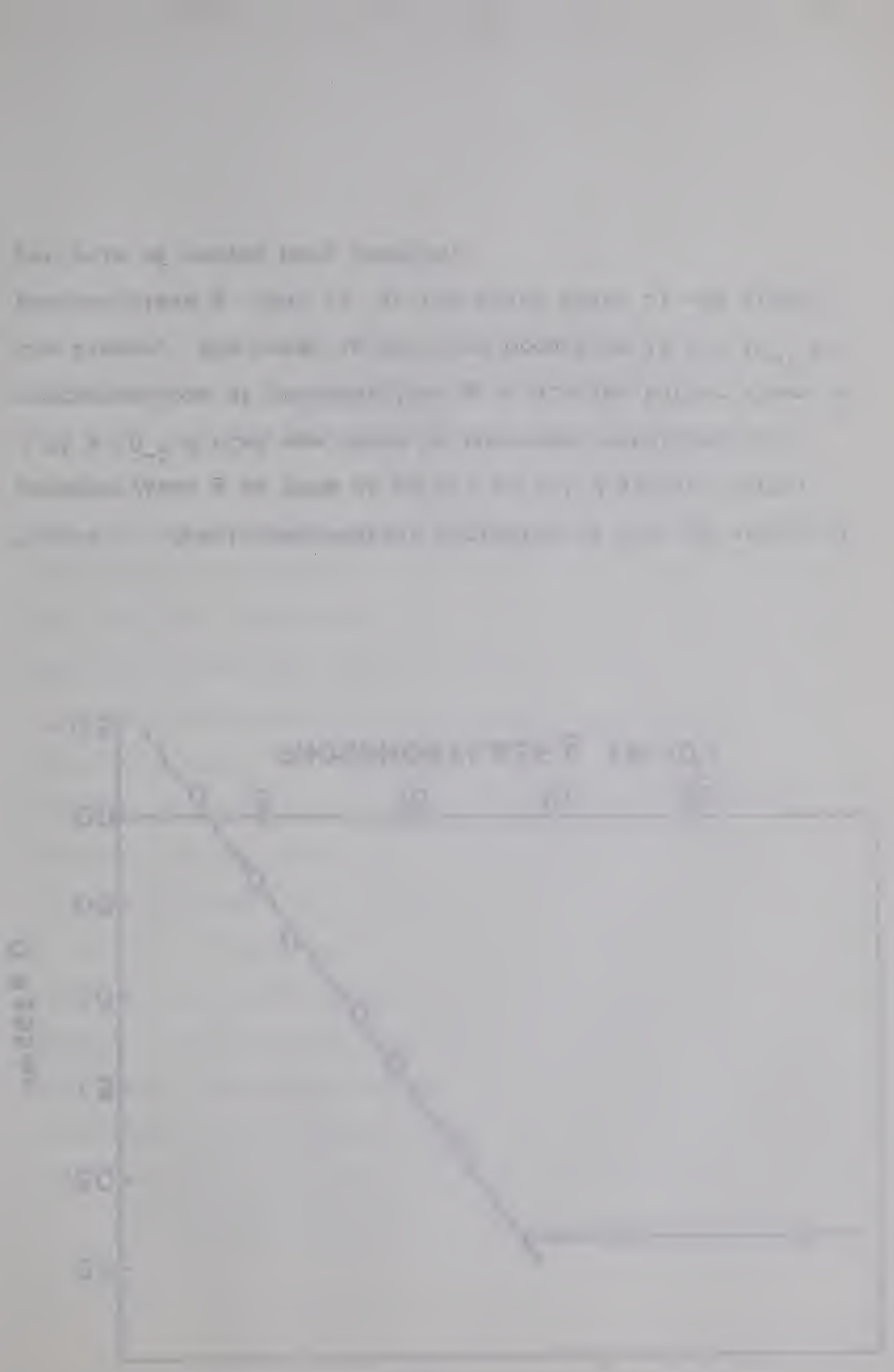


TABLE I: PCMB Titration of Phosphorylases

	Experiment	Denaturing Agent	-SH groups per Dimer (M.W. = 185,000)
I	Phosphorylase a	None	7.7
II	Phosphorylase b	None	6.6
III	Phosphorylase a	Acid	13.4
IV	Phosphorylase b	Acid	13.4
V	Phosphorylase a	SDS	11.6
VI	Phosphorylase b	SDS	13.4
VII	Phosphorylase b	Urea	12.0
VIII	Phosphorylase b	Pepsin digestion	16-18

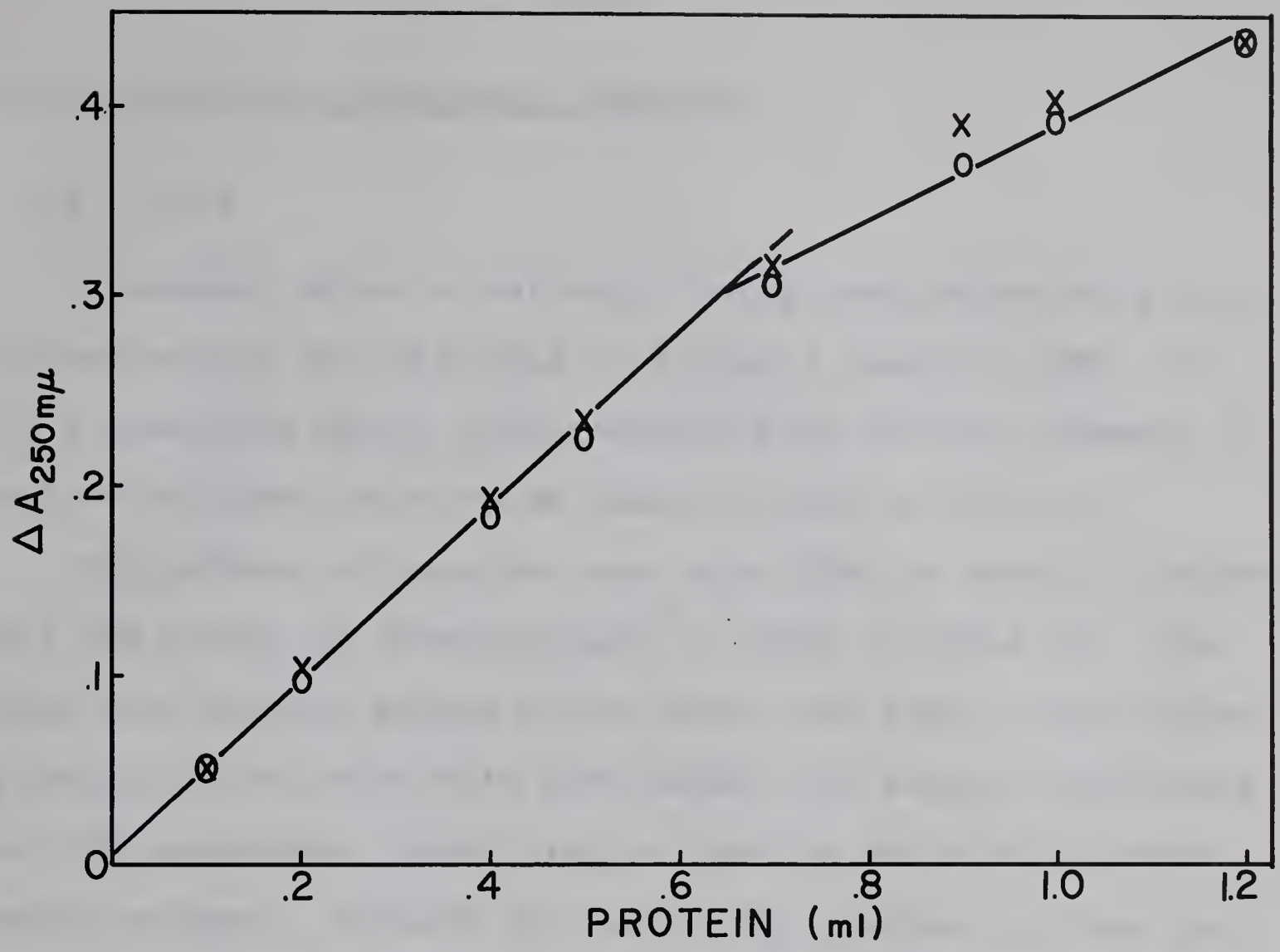
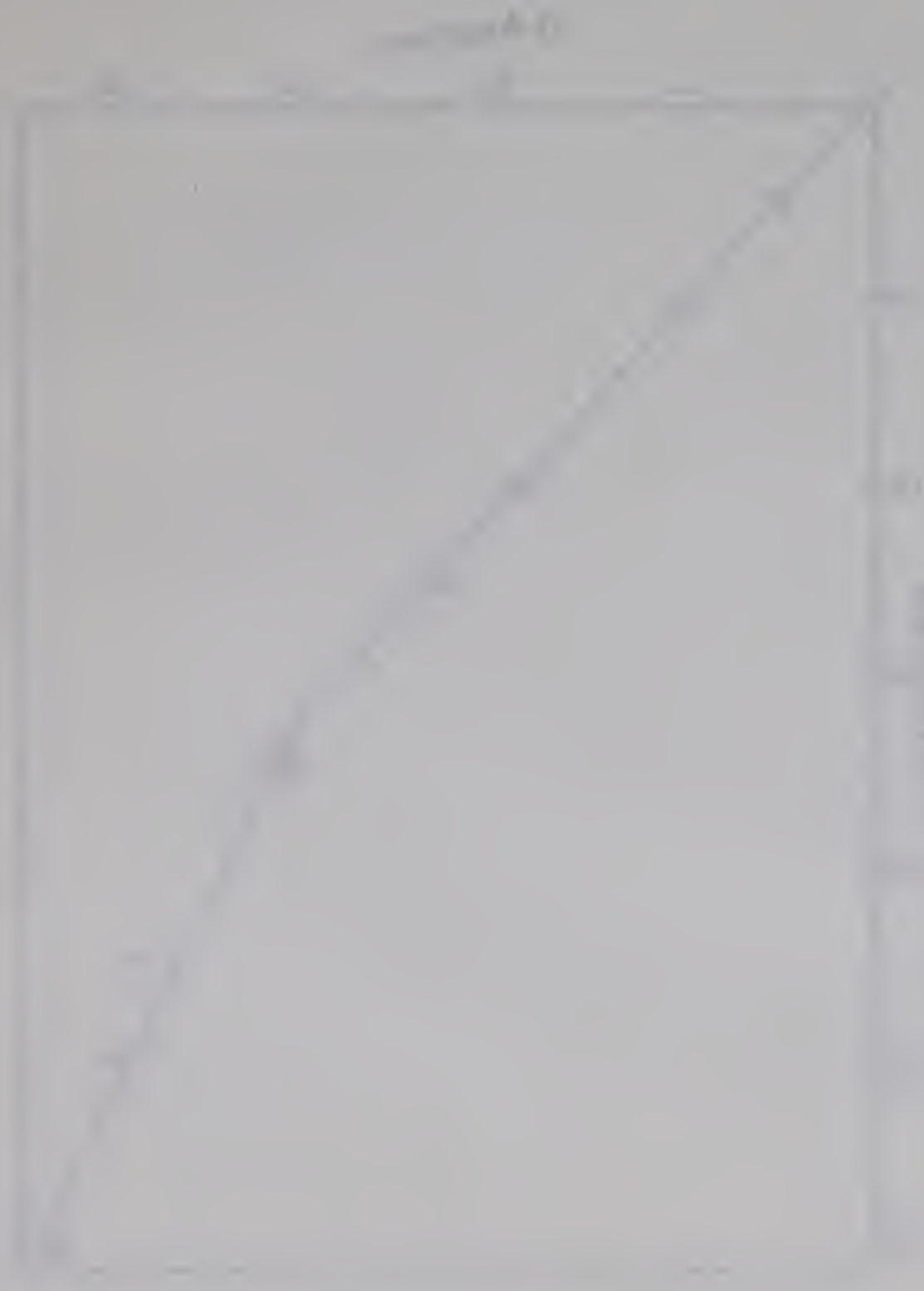


Figure 2: Spectrophotometric titration of the -SH group of phosphorylase b by PCMB after digestion of the protein with 1 mg pepsin per 10 mg phosphorylase b at 37° for 24 hours. The peptide mixture was added to cuvettes containing PCMB (5.08×10^{-5} M, final concentration) and sufficient NaOH in 0.1 M phosphate buffer to give a pH of 7.2 after addition of the peptide mixture (final volume of 2.0 ml). The concentration of the peptide mixture was 0.97×10^{-5} M. The break in the line occurs at 3.14×10^{-6} M phosphorylase b, indicating reaction of 16.2 groups per mole of protein.

O -- Change in absorbance 30 min. after beginning of reaction.

X -- Change in absorbance 60 min. after beginning of reaction.



III. Disulfide Interchange Reagents

A. DTNB

A reagent which is currently being used extensively for determination of -SH groups is Ellman's reagent, DTNB. It is a disulfide which, upon reaction with thiols, releases a yellow coloured anion which absorbs light at 412 mμ.

The pattern of reaction seen when DTNB is used to titrate the -SH groups of phosphorylase is shown in Table II. The fact that several groups which react with PCMB in the native protein do not react with DTNB under very similar conditions is not unexpected, since similar results are obtained with other enzymes. Fujioka and Snell (35) reacted 4 groups per mole of pyridoxaminepyruvate transaminase with PCMB at pH 6.9 and 6 groups per mole at pH 8.5 in the native enzyme, but could not react any groups with DTNB in the native enzyme.

There is one important difference between the conditions used for the PCMB reactions and the DTNB reactions. The addition of EDTA is the most suitable method of preventing the possible auto-oxidation of -SH groups, particularly in the denatured protein. Since EDTA was present during the DTNB reactions, it is reasonable to postulate that auto-oxidation is not the cause of the partial reaction of -SH groups with this reagent.

TABLE II: DTNB Titration of Phosphorylases

	Experiment	Denaturing Agent	-SH groups per Dimer (M.W. = 185,000)
I	Phosphorylase a	None	2.2
II	Phosphorylase b	None	3.2
III	Phosphorylase a	SDS	11.8
IV	Phosphorylase b	SDS	11.2
V	Phosphorylase b	Urea	10.2
VI	Phosphorylase b	Guanidine HCl	9.0

B. Cystine

Approximately the same number of -SH groups in the native protein react with cystine (1-2 per mole) as with DTNB (2 per mole). Phosphorylase b (4.83×10^{-6} M) was reacted with 1.78 mM ^{14}C-cystine in 50 mM Tris, 1 mM EDTA, pH 8.0 at 30°. The results were as follows:

TIME	ACTIVITY (% of zero time)	MOLES HALF-CYSTINE INCORPORATED / DIMER
0 min.	100	0
15 min.	97	1.17
30 min.	102	1.24
60 min.	98	1.21
100 min.	108	1.28
3 hours		1.33
21 hours	95	3.13
*21 hours	95	3.11

*The protein was put onto a Sephadex G-25 Gel-filtration column equilibrated with 0.5 M Tris, 10 mM EDTA, pH 8.0. After elution from the column, a sample of protein (same concentration as previously) was assayed for activity and radioactivity.

The results of the work on both native and denatured protein are shown in fig. 3. Because of the smaller size of cystine, it was anticipated that it would react with at least as many -SH groups as DTNB. As can be seen, this is

not the case since 10.2 groups reacted with DTNB in 8 M urea while only 7 groups reacted with cystine under the same conditions. In addition, it appears that the presence of a small concentration of mercaptoethanol results in a dilution of the labeled cystine on the protein with time. Although 7 moles of half-cystine is incorporated per mole of phosphorylase b within 15 minutes in the presence of 8 M urea, this value drops to 4.3 moles per mole by 8 hours when mercaptoethanol is present in the reaction mixture. When the protein has been subjected to a preliminary denaturing treatment with hydrochloric acid, the approach to the equilibrium disulfide mixture is accelerated as indicated by a more rapid decrease in the ^{14}C-cystine content of the protein.

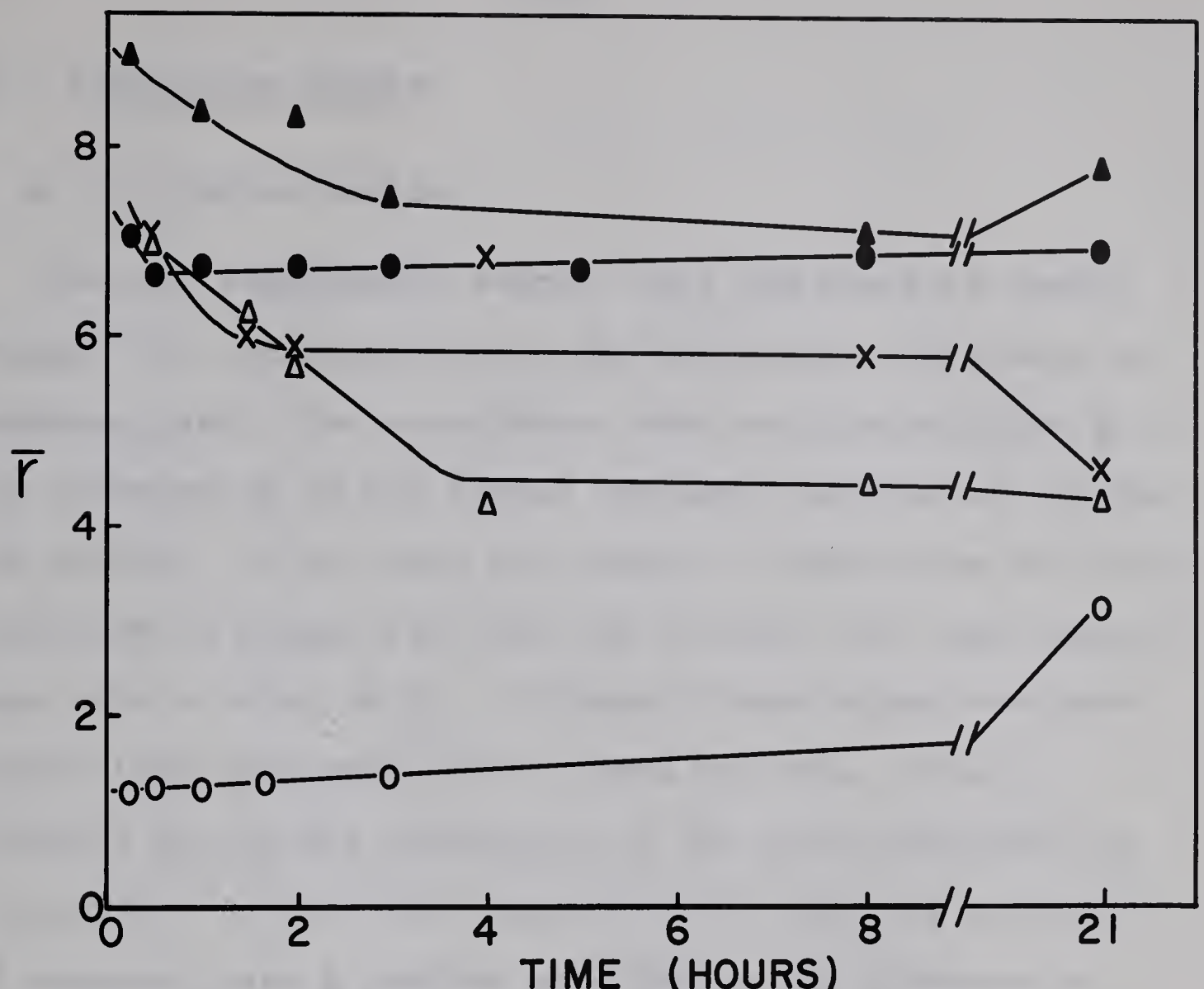


Figure 3: Incorporation of ^{14}C-cystine into phosphorylase b.

$\bar{r}$ -- moles of half-cystine incorporated per mole of enzyme.

O -- 50 mM Tris, 1 mM EDTA, pH 8.0. 1.78 mM cystine.

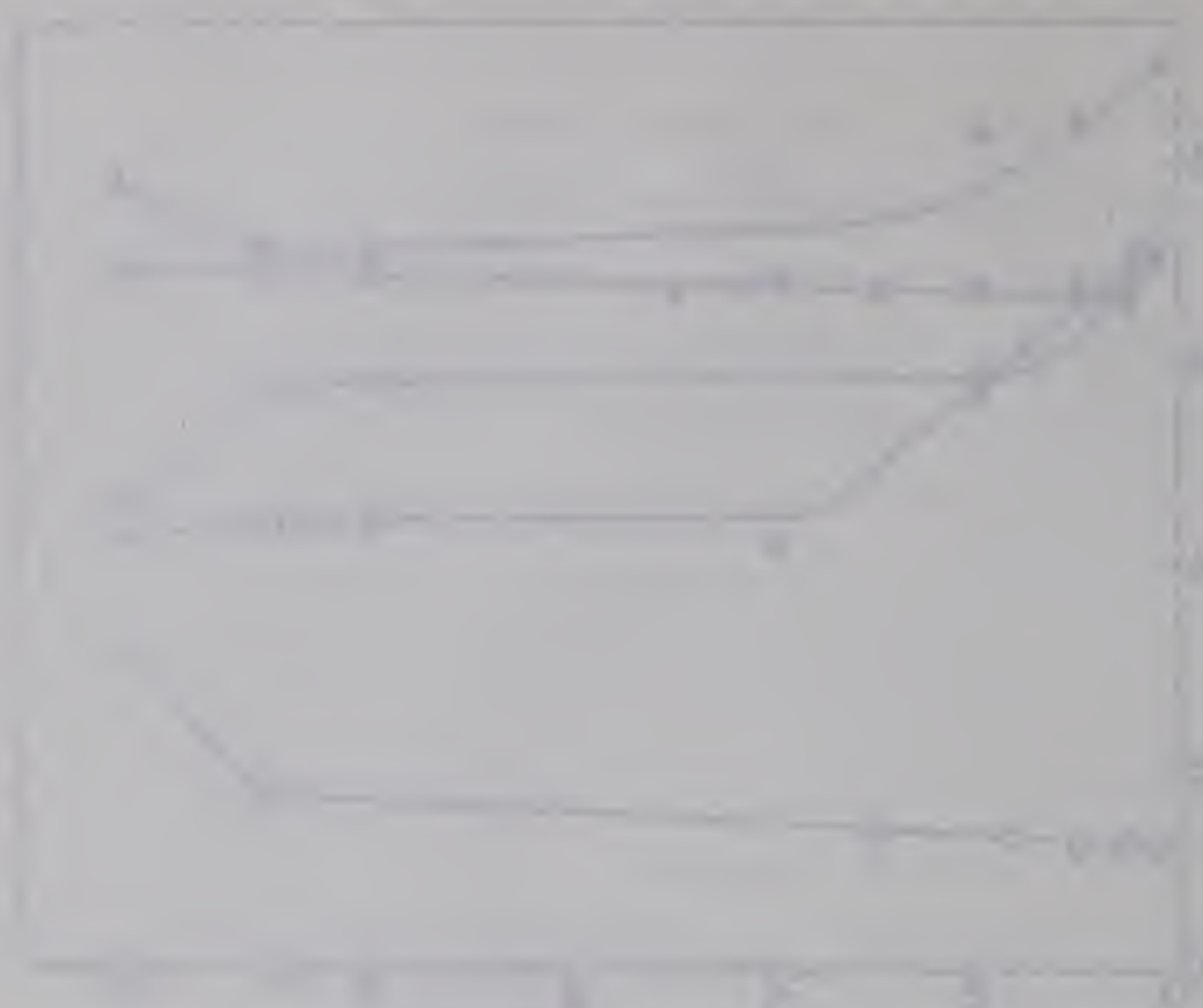
Δ -- 1 mM KCl, 0.1 mM mercaptoethanol, 1.1 mM EDTA, 5 mM Tris, 8 M urea, pH 8.0; sufficient HCl was added to the protein to lower the pH to 2 before the addition of urea. 2.14 mM cystine.

X -- 1 mM KCl, 0.1 mM mercaptoethanol, 1.1 mM EDTA, 5 mM Tris, 8 M urea, pH 8.0. 2.14 mM cystine.

● -- 50 mM Tris, 1 mM EDTA, 8 M urea, pH 8.0. 1.66 mM cystine.

▲ -- 50 mM Tris, 1 mM EDTA, 5 M guanidine HCl, pH 8.0 2.00 mM cystine.

Protein concentration: 4.83×10^{-6} M. Temperature, 30°.



IV. Alkylating Agents

A. N-ethylmaleimide

Several experiments suggest that NEM may be a useful reagent for investigation of the unreactive -SH groups of phosphorylase. Two experiments done on phosphorylase a in the presence of 1% SDS showed complete reaction of all the -SH groups. In one case the change in absorbance at 310 mμ indicated 43 groups per mole had reacted; the other experiment gave a value of 41. Although these values are both higher than the total of 36 groups per mole, this is probably due to the inaccuracy of the spectrophotometric titration. In two other experiments, 11 groups per mole of phosphorylase b reacted with NEM in the presence of glycine buffer at pH 2.2. Since ^{14}C-NEM is now available, incorporation of labeled reagent would be the preferred method of measurement. Where there is doubt about the groups reacting, the amount of S-succinyl-cysteine present after acid hydrolysis could be measured.

In another experiment, phosphorylase b was first reacted with a slight excess of PCMB over the reactive -SH groups. The NEM, labeled in the C-1 position of the ethyl group was added after one hour. As can be seen in fig. 4, incorporation to the extent of 12 groups per mole took place immediately in the protein which had been pretreated with PCMB. There was only a small amount of incorporation into the protein which had not been treated with PCMB. Addition of

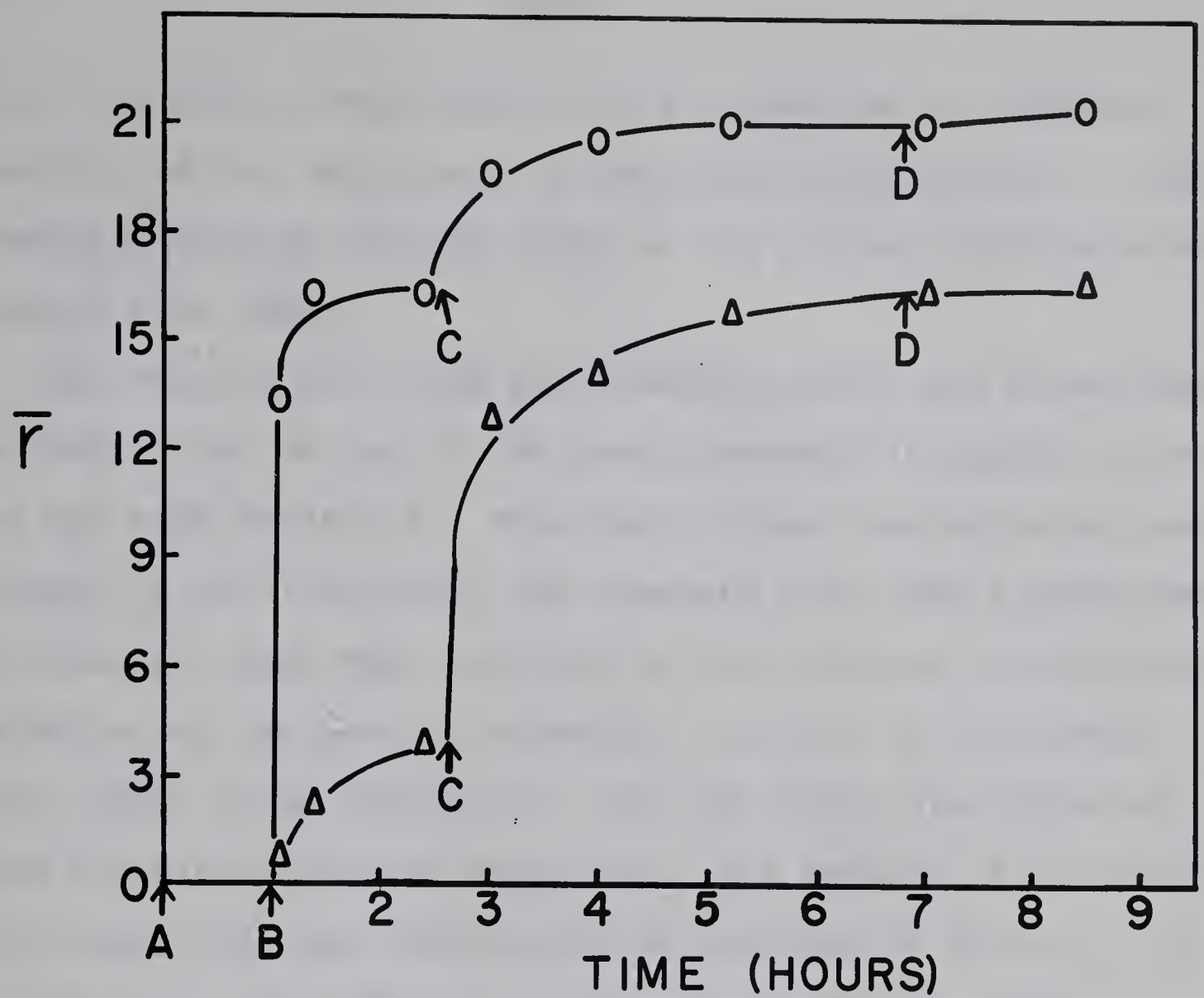
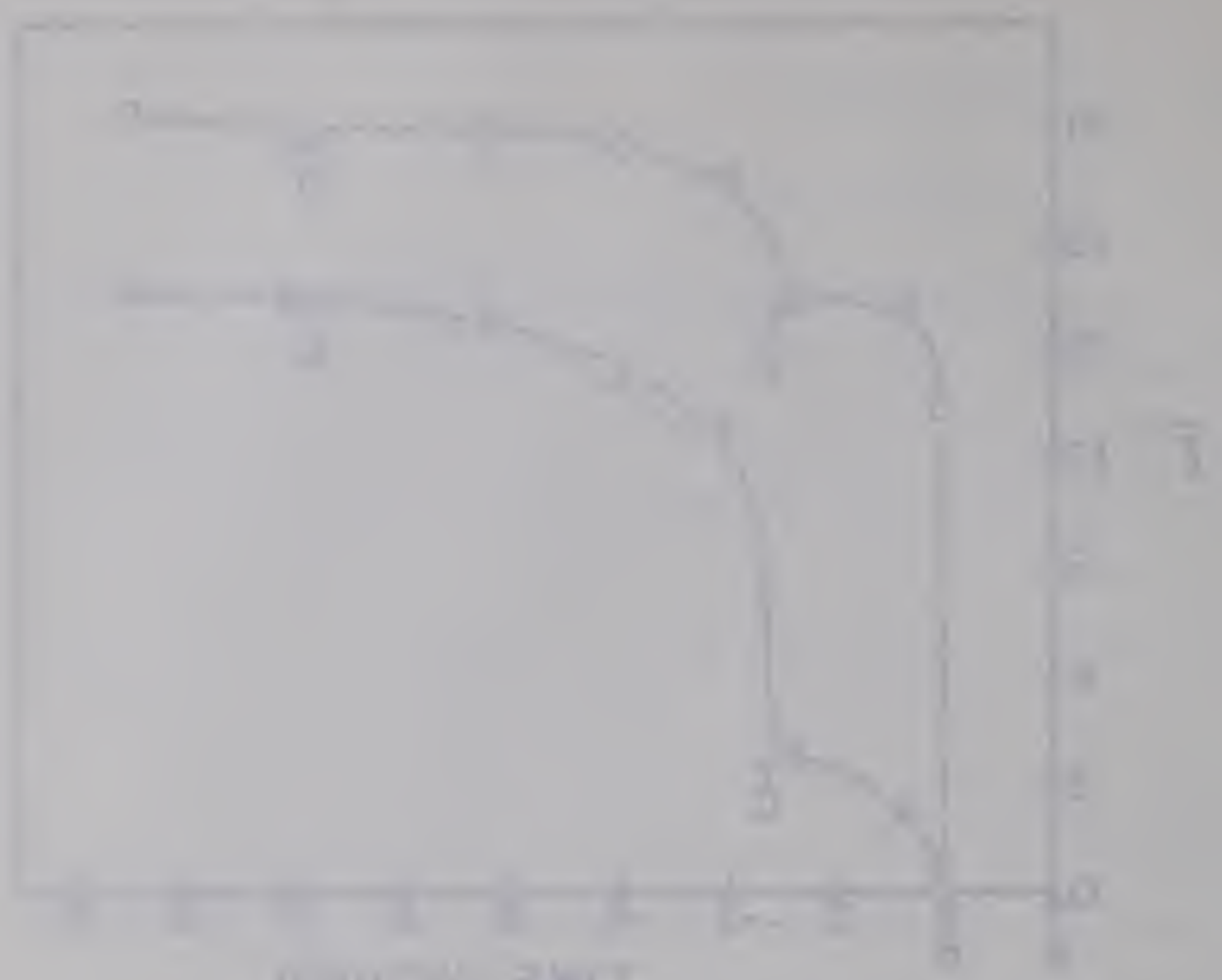


Figure 4: Incorporation of NEM into phosphorylase b. 2.00×10^{-5} M phosphorylase b in 0.25% KCl, 5 mM sodium glycerophosphate, pH 7.0. At zero time, PCMB was added to give the specified concentration of protein and 17.0×10^{-5} M PCMB. At "B", N-ethyl-1-^{14}C-maleimide was added to a concentration of 9.32×10^{-3} M. Crystalline urea was added to 8 M at "C". The pH was adjusted to 2.0 by addition of 1N HCl at "D". Temperature, 30°.

$\bar{r}$ -- moles of ^{14}C-NEM incorporated per mole of phosphorylase b.

O -- protein treated with PCMB.

Δ -- no PCMB added.



urea to a final concentration of 8 M resulted in complete labeling of the -SH groups in the pre-treated protein. This complete labeling did not occur in the protein not previously treated with PCMB.

The results with PCMB pre-treated protein are noteworthy in that 15 out of the 18 -SH groups reacted in protein that had not been denatured. This has not been accomplished previously in our laboratory and suggests that PCMB potentiates the reaction with NEM, possibly by facilitating an extensive unfolding of the protein molecule. Further it is possible that, under these conditions, the -SH groups are protected from any auto-oxidation reactions. The results of an experiment using the same combination of reagents is shown in fig. 5. In this case, the PCMB was radioactive while the NEM was unlabeled. As was expected, 6 moles of PCMB was incorporated per mole of phosphorylase b initially; however, when the NEM was added, no PCMB was released from the protein. When urea was added, some loss of PCMB occurred, but not to the extent expected. About 95% of the PCMB present was incorporated into the protein which had not had NEM added after the urea was added.

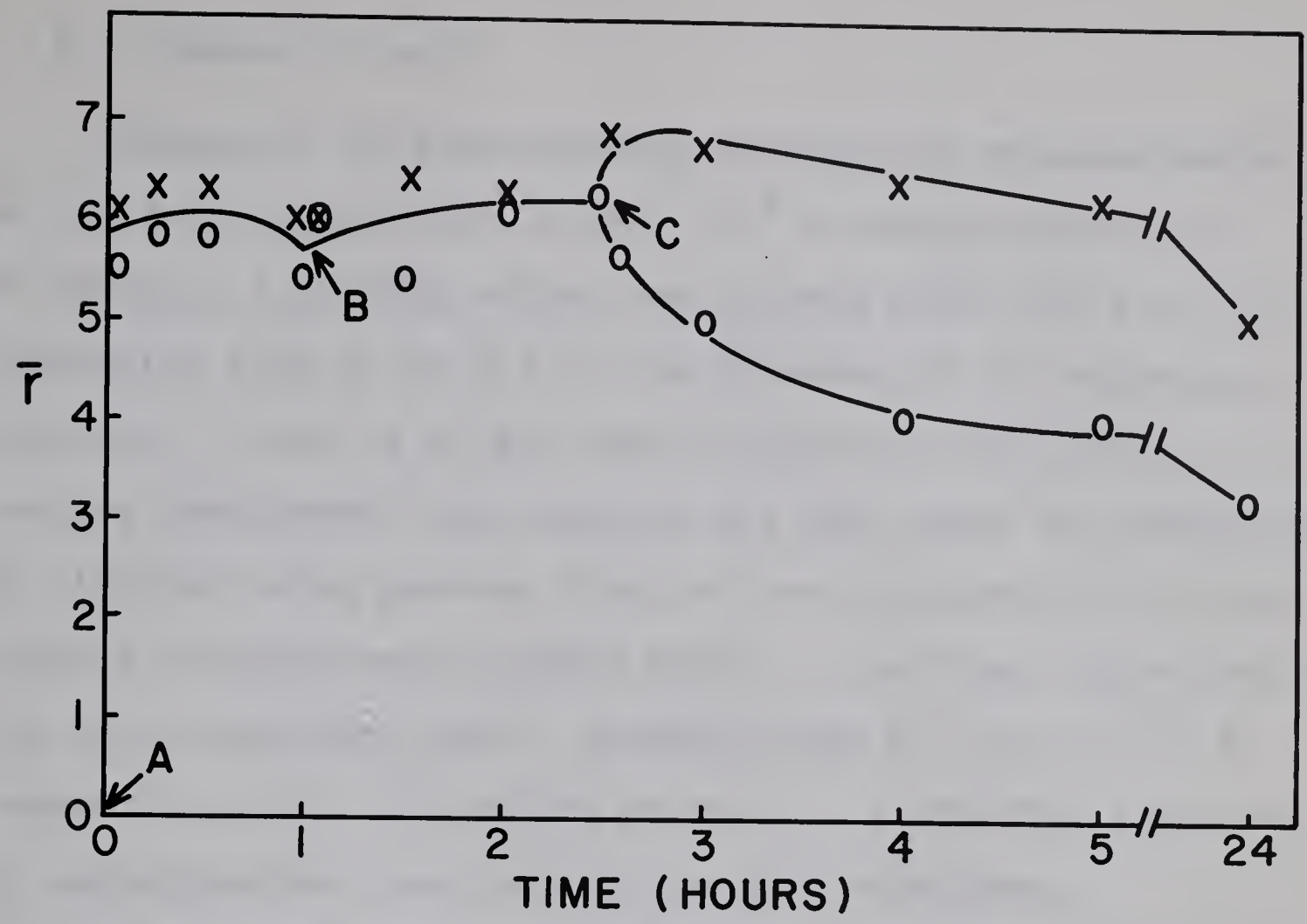
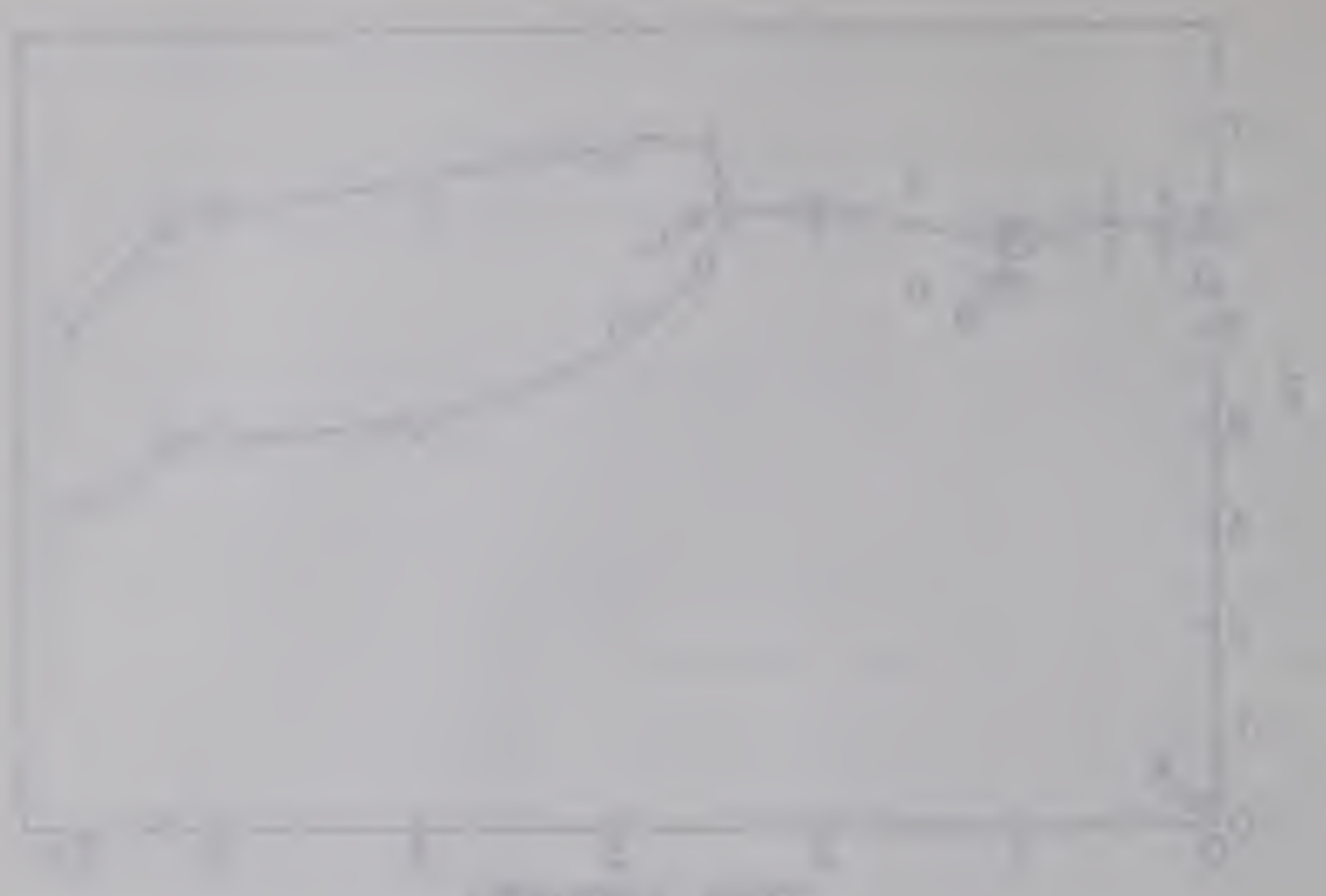


Figure 5: Effect of NEM on incorporation of PCMB. "A". ^{14}C-PCMB was added to a concentration of 1.61×10^{-4} M to 2.29×10^{-5} M phosphorylase b in 1% KCl, 1% sodium glycerophosphate, pH 7.5 at zero time. NEM was added to a concentration of 10^{-2} M at "B". At "C" crystalline urea was added to a concentration of 8 M.

$\bar{r}$ -- moles of ^{14}C-PCMB incorporated per mole of phosphorylase b.

O -- NEM added.

X -- no NEM added.



B. Iodoacetic Acid

Iodoacetic acid was used in another set of experiments. In the first experiment, 4.88×10^{-6} M phosphorylase b in 50 mM Tris, 1 mM EDTA buffer was reacted with 2.75×10^{-3} M iodoacetic acid at pH 8.2 in the presence of 0.1 mM mercaptoethanol. Urea (8 M) was used to denature the protein. In another experiment, the reaction was done under an atmosphere of nitrogen using protein that had been dialyzed for 3 hours against nitrogen-equilibrated buffer. The final concentrations for this experiment were: phosphorylase b, 5.56×10^{-6} M; iodoacetic acid, 3.17 mM; 50 mM Tris; 1.5 mM EDTA; 8 M urea. No mercaptoethanol was present in this experiment.

The reaction was measured both by incorporation of ^{14}C label and by determination of carboxymethylcysteine after acid hydrolysis. Of the half-cystines in phosphorylase b, 14.8 were recovered as carboxymethylcysteine in the mercaptoethanol protected system at 24 hr, and slightly less (12.2) in the nitrogen equilibrated system after 24 hours of reaction. Some non-specific incorporation took place as indicated by the incorporation of up to 21 moles of the labelled reagent in the mercaptoethanol protected system in the same 24 hour reaction period. It is noteworthy that both of the alkylating agents discussed so far, iodoacetic acid and NEM, reacted to about the same extent when the protein was denatured with urea, close to 14 groups per mole reacting in each case.

C. Iodoacetamide

1) Effect on the structure and activity of phosphorylase b

Iodoacetamide was found to react with the same number of -SH groups in native protein as PCMB. Since the reaction is much slower, a large excess of reagent is required. When 5 - 10 mg per ml of phosphorylase b is reacted with 10 mM iodoacetamide at 30°, 85% of the activity is lost in about 8 hours. That the reaction of iodoacetamide is faster and more extensive than that of iodoacetic acid is not surprising since this is usual with these two reagents (1). A pseudo-first order plot of the activity loss is shown in fig. 6. The notable feature is that the reaction is linear to beyond 95% reaction. This result is highly reproducible.

As occurs with PCMB, the reaction of iodoacetamide results in a conversion to monomer. Mercaptoethanol was used to stop the reaction at various times. After centrifuging out the precipitate, the samples were subjected to ultracentrifugal analysis and the percentage of various components determined by area analysis. The 32 minute pictures are shown in fig. 7. In fig. 8 the percentage dimer remaining is plotted on a semi-logarithmic scale along with the inactivation reaction. The conversion of dimer follows pseudo-first order kinetics, but the reaction appears to be much slower than the inactivation reaction. Since an extensive amount of precipitation occurs during this reaction,

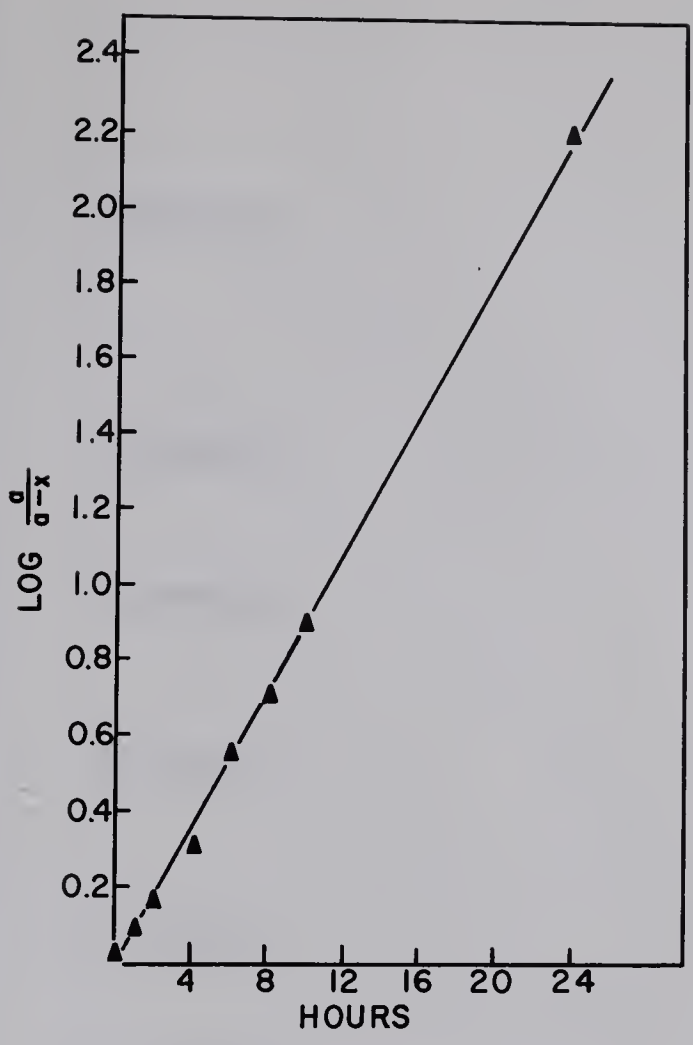


Figure 6: Inactivation of phosphorylase b with iodoacetamide. 4.83×10^{-5} M phosphorylase b in 20 mM sodium glycerophosphate, 1% KCl, 1.5 mM EDTA, pH 7.5 was reacted with 10.3 mM iodoacetamide at 30°. At the times shown samples were diluted 0.1 ml + 0.9 ml of buffer, then 0.1 ml of the 1/10 dilution + 1.4 ml of buffer. The buffer for dilution was 20 mM sodium glycerophosphate, 50 mM mercaptoethanol, 1.5 mM EDTA, pH 6.8. 0.1 ml was used for activity determination as specified in Methods. The half-time for this particular reaction was 3.3 hours. The pseudo-first order constant was 3.5×10^{-3} min^{-1}.

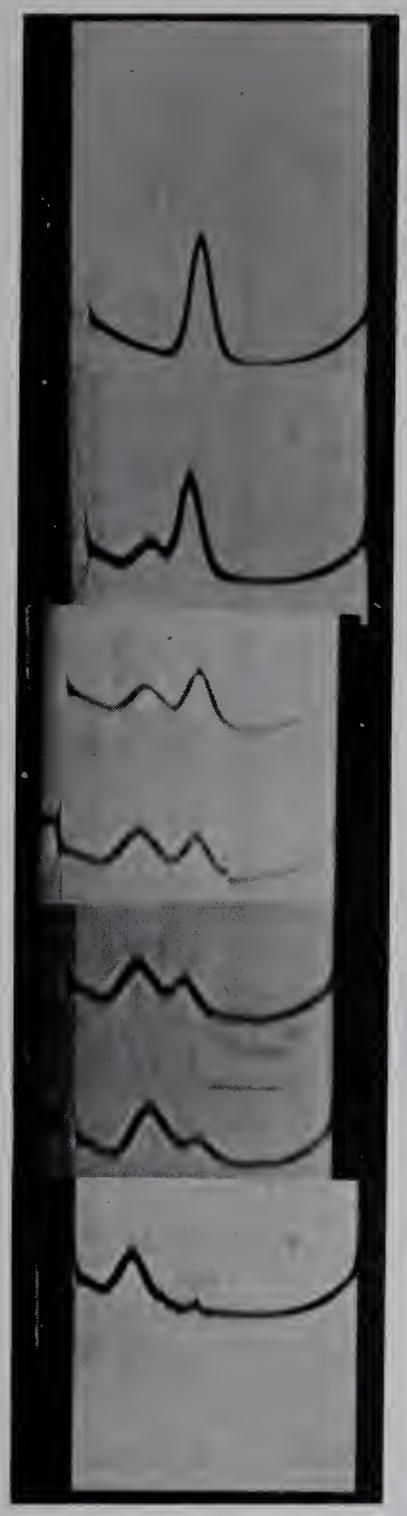


Figure 7: Effect of iodoacetamide on the structure of phosphorylase b. Reaction of 4.83×10^{-5} M phosphorylase b in 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5 with 10.0 mM iodoacetamide. Reaction stopped by addition of mercaptoethanol, to a concentration of 0.5 M, to samples taken at the specified times. The precipitated protein was spun down, then 6 mg per ml of the supernatant assayed in the ultracentrifuge. 32 minute pictures. The average S_{20} values were 8.6 for the fast component and 5.3 for the slow component.

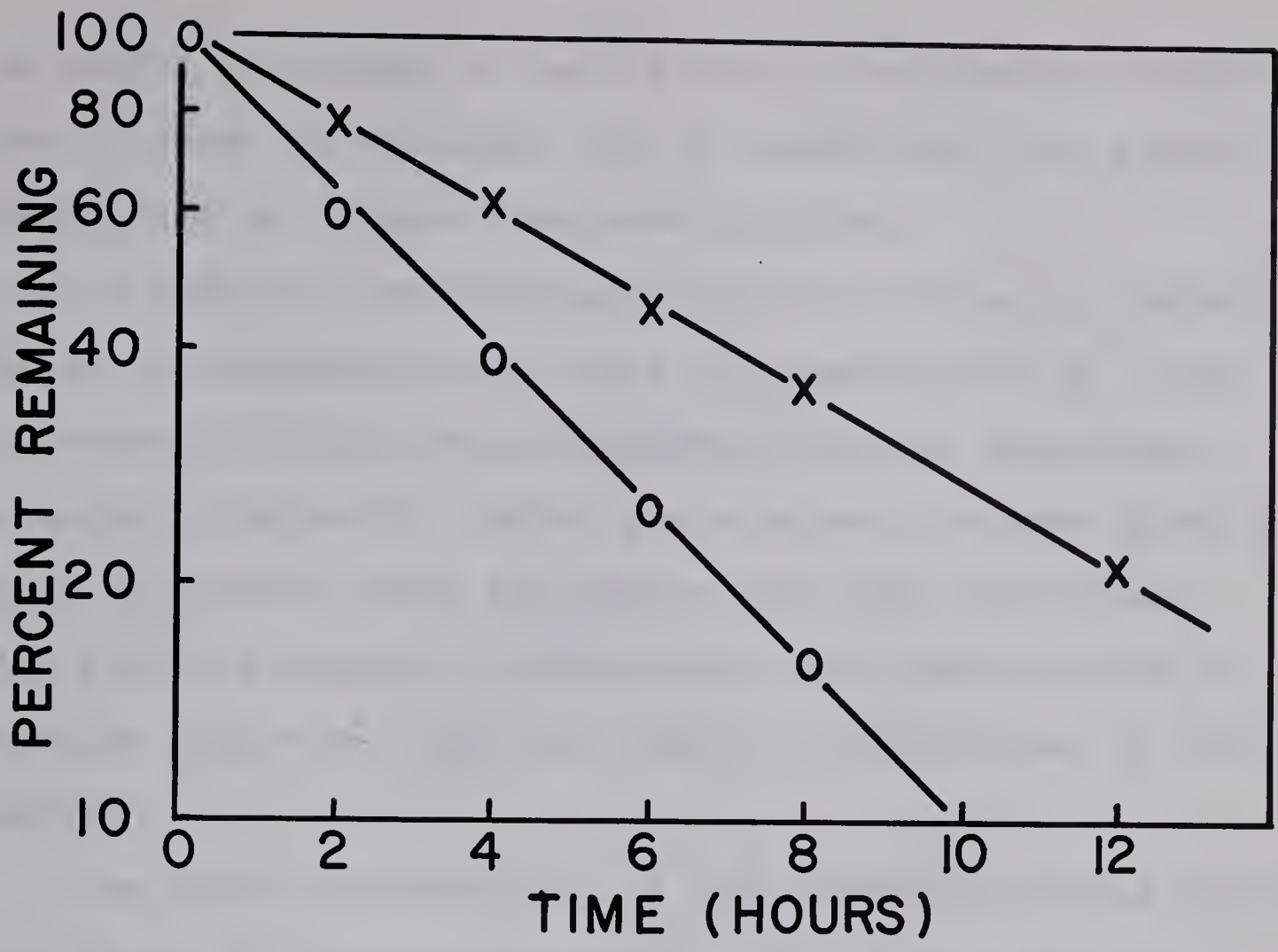
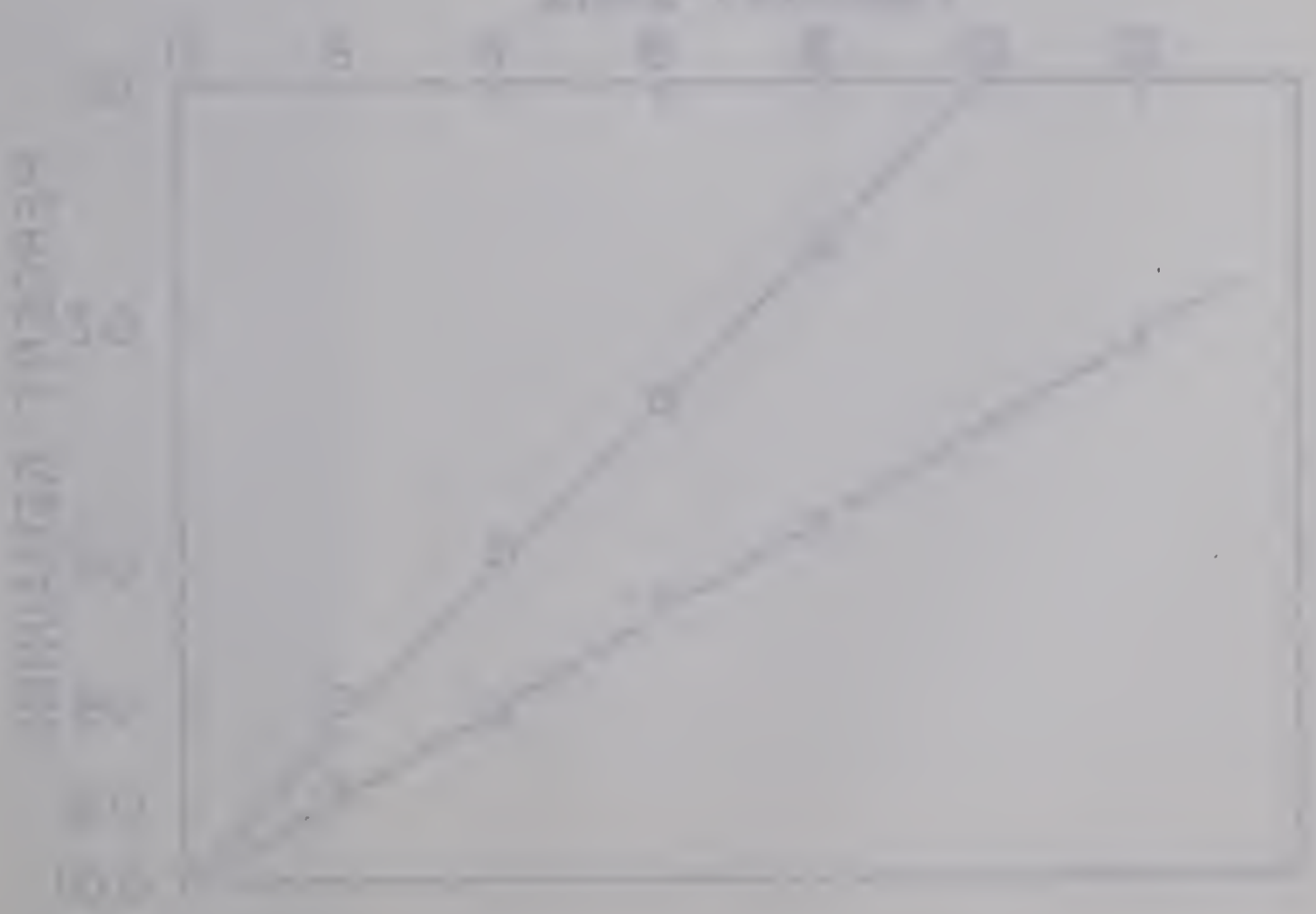


Figure 8: Semi-logarithmic plot of inactivation and dissociation reactions. Conditions specified in fig. 7. The percentage dimer remaining was calculated from the areas under the peaks. Activity determinations were done as previously specified (fig. 5). The half-time for this inactivation reaction was 3.0 hours.

O -- activity remaining.

X -- amount of dimer remaining.



the amount of monomer at each time must be somewhat larger than is shown by the graph (it is assumed that the protein dissociated to monomer then precipitates).

At 8 hours of reaction approximately 6 moles of iodoacetamide is incorporated per mole of phosphorylase b. When the number of moles of carboxymethylcysteine formed was measured, a value of 7 moles per mole was obtained after 8 hours of reaction when the protein was 85% inactivated. This provides chemical confirmation that iodoacetamide is reacting only with -SH groups under the conditions of this reaction.

The total incorporation of iodoacetamide plotted against time shows an interesting result. Fig. 9 is a plot of incorporation into phosphorylase b. It appears that after reaction of a first group of about 6 -SH, a subsequent reaction takes place at a rate which is of the same order of magnitude as the overall rate of the first group. However, these results are difficult to interpret.

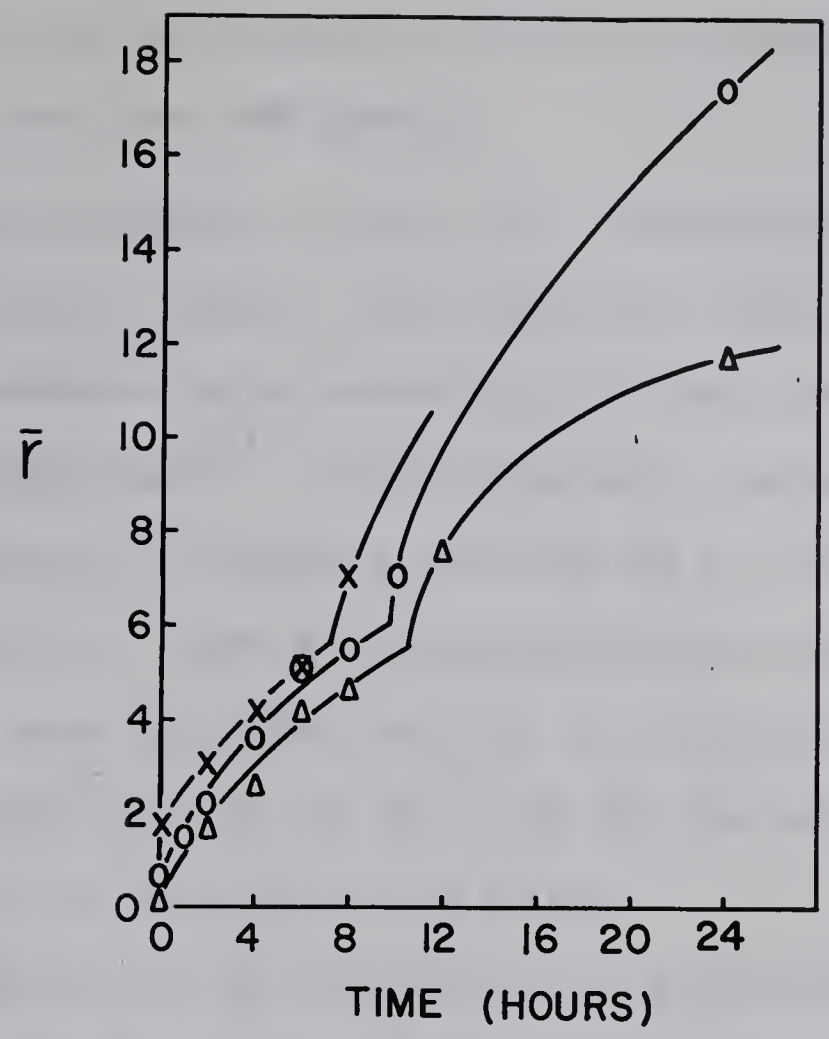


Figure 9: Incorporation of iodoacetamide into phosphorylase b with time. Values taken from three experiments on phosphorylase b. Conditions: 9 - 10 mg per ml protein, 10 mM iodoacetamide, 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5.

$\bar{r}$ -- moles of ^{14}C-iodoacetamide incorporated per mole of phosphorylase b.

2) Isolation and identification of peptides containing the reactive -SH groups

Since approximately 6 moles of iodoacetamide was incorporated per mole of dimer, this suggested that 3 cysteine residues per monomer were reacting. It was possible to identify the peptides[1] containing each particular -SH group by scanning a ninhydrin stained pH 6.5 electropherogram for radioactivity. Such an electropherogram along with its radioactivity scan is shown in fig. 10, where the positions of the major peptides (A, N, B1, and B2) as well as several minor peptides (C, D, and E) are marked.

A large quantity of phosphorylase b was alkylated with iodoacetamide for 8 hours. To the 85% inactivated enzyme, which was partially precipitated, concentrated formic acid was added to a final concentration of 5%. After centrifuging out the insoluble precipitate, the protein was exhaustively dialyzed against 5% formic acid, then digested with pepsin for 24 hours at 37°. The peptide mixture was separated on paper by high voltage electrophoresis at pH 6.5.

The radioactive sections were cut out and oxidized over performic acid, then subjected again to electrophoresis at pH 6.5, then 1.8, then 3.5. The position of the radioactive peptides was determined after each step by scanning

[1]The peptides were obtained by a pepsin digestion of ^{14}C-iodoacetamide treated phosphorylase b, as described below.

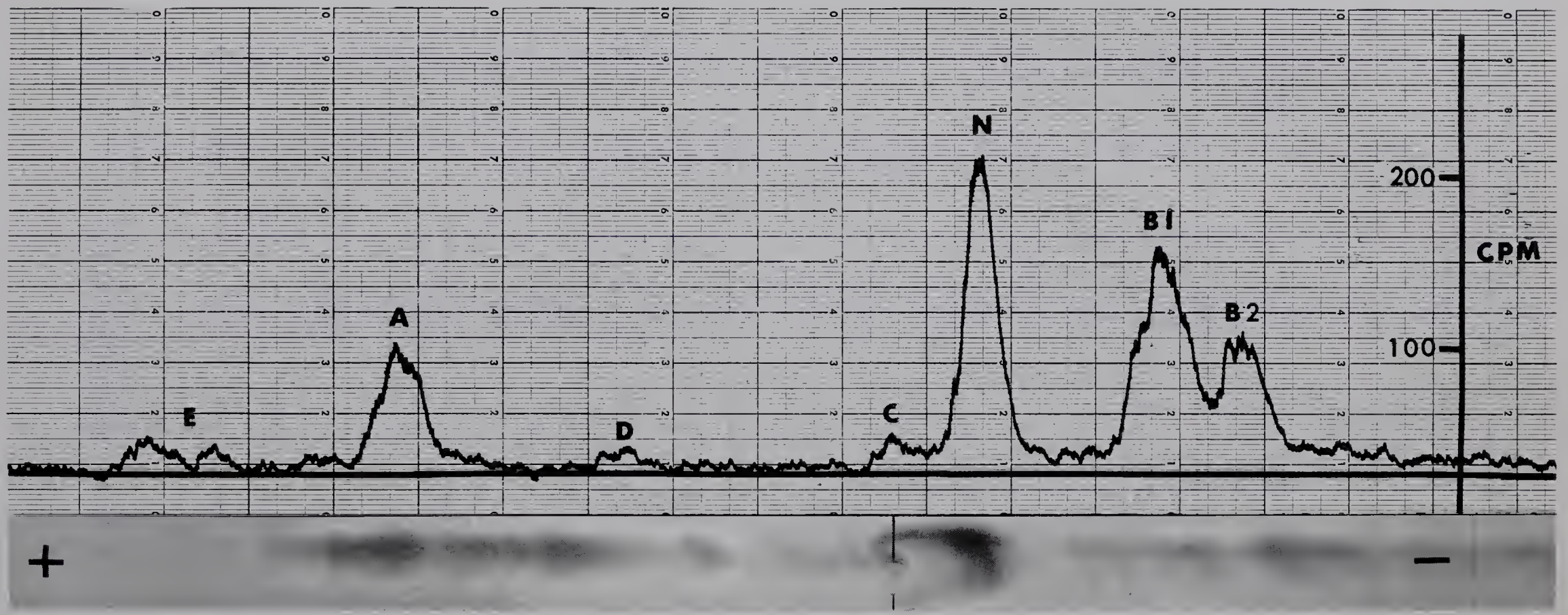

N
B1
B2
A
E
D
C
200
100
CPM
+
−

Figure 10: Position of peptides containing reactive -SH groups after pH 6.5 electrophoresis. Phosphorylase b or phosphorylase a was treated as specified in fig. 5. Mercaptoethanol was added in a 50-times excess to stop the reaction. The sample was made 5% with respect to formic acid 30 minutes after addition of mercaptoethanol. The sample was then dialyzed for 36 hours against 5% formic acid. Pepsin was added (1 mg per 10 mg phosphorylase) and the sample incubated for 24 hours at 37°. 0.6 mg was applied to a one cm diameter spot on #1 paper, then electrophoresis carried out for one hour at 3000 volts. A strip was then cut out which included one cm on either side of the original spot to include the entire 0.6 mg of protein. The strip was put through the Actigraph III scanner at 15 cm per hour. The peptides containing radioactivity are labeled on the strip scan.

side strips. The purified peptides were then eluted from the paper and subjected to amino acid analysis. Two peptides were readily obtained by this method. The peptide which occurred at the neutral position upon pH 6.5 electrophoresis contained 2 equivalents of alanine, 1 of phenylalanine, and 1/2 of cysteic acid.[2] (The carboxyamidomethyl cysteine residue is known to be unstable to oxidation and to hydrolysis, and would therefore be recovered in low yield as cysteic acid.) Analysis indicated that alanine was the N-terminal amino acid. On this basis the peptide was matched with one of the -SH containing peptides whose sequence had been determined by Mr. C. Zarkadas (42).

N peptide ala-cys-ala-phe-

The second peptide isolated contained two residues of aspartic acid, one of which was established to be asparagine, as well as one residue of alanine in addition to the cysteine residue.[2] Again, the cysteine residue was recovered in low yield as cysteic acid. The N-terminal residue was asparagine and the sub-terminal, alanine; matching the peptide whose sequence had been determined by Mr. Zarkadas.

A peptide aspNH_2-ala-cys-asp-

Both the peptides isolated contained some impurities, but these were not felt to be important since the yield in μmoles matched the amount expected on the basis of the radioactivity present before hydrolysis.

The N peptide was also isolated as a tripeptide, without

[2]Footnote on page 35.

the C-terminal phenylalanine residue. As such, it is separated from the peptide shown when subjected to electrophoresis at pH 3.5 because of a slightly different pKa of the terminal carboxyl group and because of a large difference in molecular weight. After electrophoresis of a digest treated as described, a third radioactive area (B1, B2) is seen upon pH 6.5 electrophoresis (fig. 10). The peptides involved are slightly basic. We have been unable to isolate the peptides involved in sufficient purity or sufficient quantity to determine the composition or sequence. The work done indicated that the two peptides involved are very large (up to 25 amino acid residues). The two peptides can be partially separated by electrophoresis for a slightly extended period of time (60 minutes instead of 50) at pH 6.5, or by electrophoresis at pH 3.5 following 6.5. Fig. 11 is a diagram of the positions of the peptides specified after two dimensional electrophoresis. The positions of the radioactive peptides were determined by exposing the electropherogram to X-ray film for about 4 weeks.

[2]Amino acid analysis of peptides.

Amino acid	N peptide (μmoles)	A peptide (μmoles)
Aspartic acid		0.0110*
Alanine	0.0108	0.0060
Phenylalanine	0.0055	
Cysteic acid	0.0022	0.0017

*N-terminal established by Mr. Zarkadas to be asparagine.

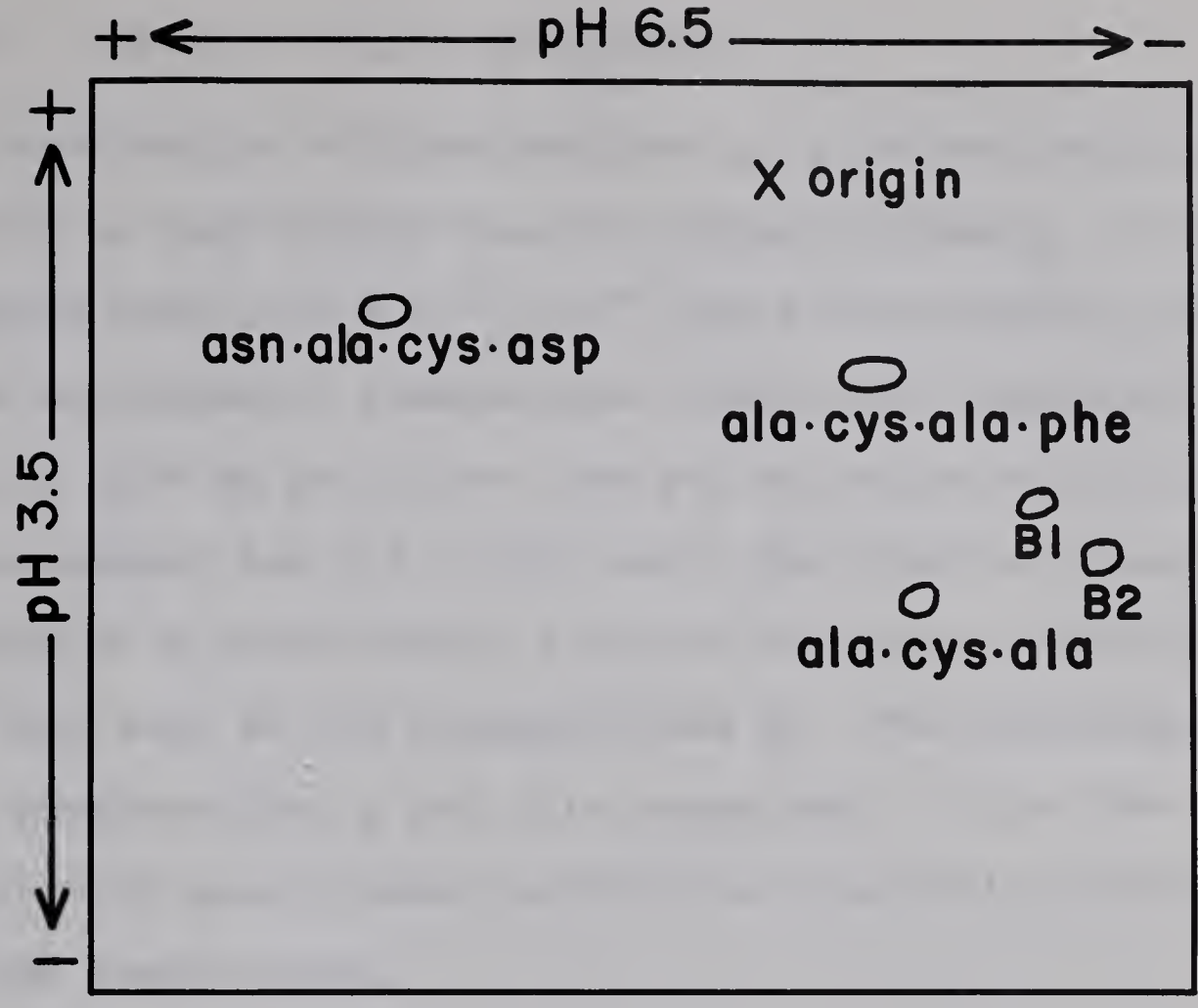


Figure 11: Two-dimensional analysis of iodoacetamide labeled peptides. Protein reacted with iodoacetamide-1-^{14}C as specified in fig. 5. Peptide mixture prepared as described in fig. 9. After electrophoresis at pH 6.5, the mixture was subjected to electrophoresis at pH 3.5, for 60 minutes, at 3000 volts. The sheet was then trimmed to 14 x 17 inches and placed next to Kodak Blue Brand X-Ray film for up to 4 weeks depending on the specific activity of the peptide mixture.

3) Reaction of phosphorylase *a*

Inactivation of phosphorylase *a* by iodoacetamide was found to be much faster than for phosphorylase *b*. The rate constants were 12.9×10^{-3} min^{-1} for phosphorylase *a* (average of two experiments, pseudo-first order rate constant for reaction of 6 mg per ml or 9 mg per ml enzyme with 10 mM iodoacetamide) and 3.4×10^{-3} min^{-1} for phosphorylase *b* (average of 4 experiments, 9 mg per ml enzyme, other conditions the same as for phosphorylase *a*). The advantage of using phosphorylase *a* for this experiment is that the formation of precipitated protein is relatively slower than the inactivation.

4) Kinetics of specific -SH groups

In an attempt to correlate the inactivation with reaction of specific peptides, the kinetics of incorporation into the peptides were studied during inactivation. Mercaptoethanol was used to stop the reaction in samples taken at various times (reaction conditions were the same as previously specified). After analysis according to the legend for fig. 10, the area under the peak for each peptide was determined. A direct plot of the areas determined for phosphorylase a is shown in fig. 12.

It appears that the B1 and B2 -SH groups react rapidly, compared to the other -SH groups. In this experiment, the total extent of their reaction took place within the 30 min. before the first sample was taken. The pattern of labeling for the C peptide is similar to that of the B1 and B2 peptides. The N and A peptides are being labeled progressively during the course of this reaction suggesting that these two peptides (but not the B1 or B2 peptides) are concerned with the inactivation reaction. This will be discussed more fully later. The E peptide and the D peptide, which are evident only during the later part of the reaction, have a labeling pattern that resembles that of the A or N peptides rather than that of the B peptides.

As has previously been stated the evidence indicates that a total of 3 -SH groups per monomer are reacting with iodoacetamide. If we assume that a total of three peptides

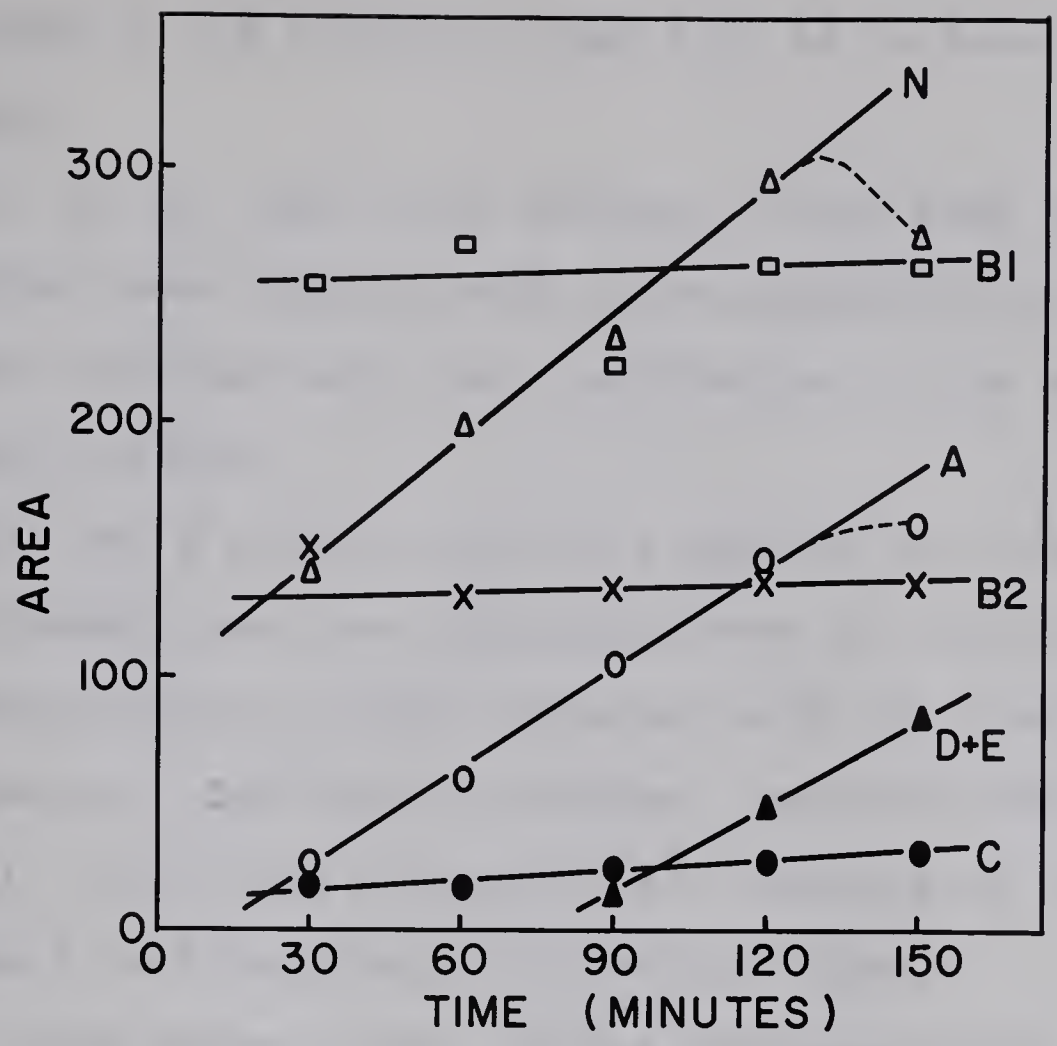
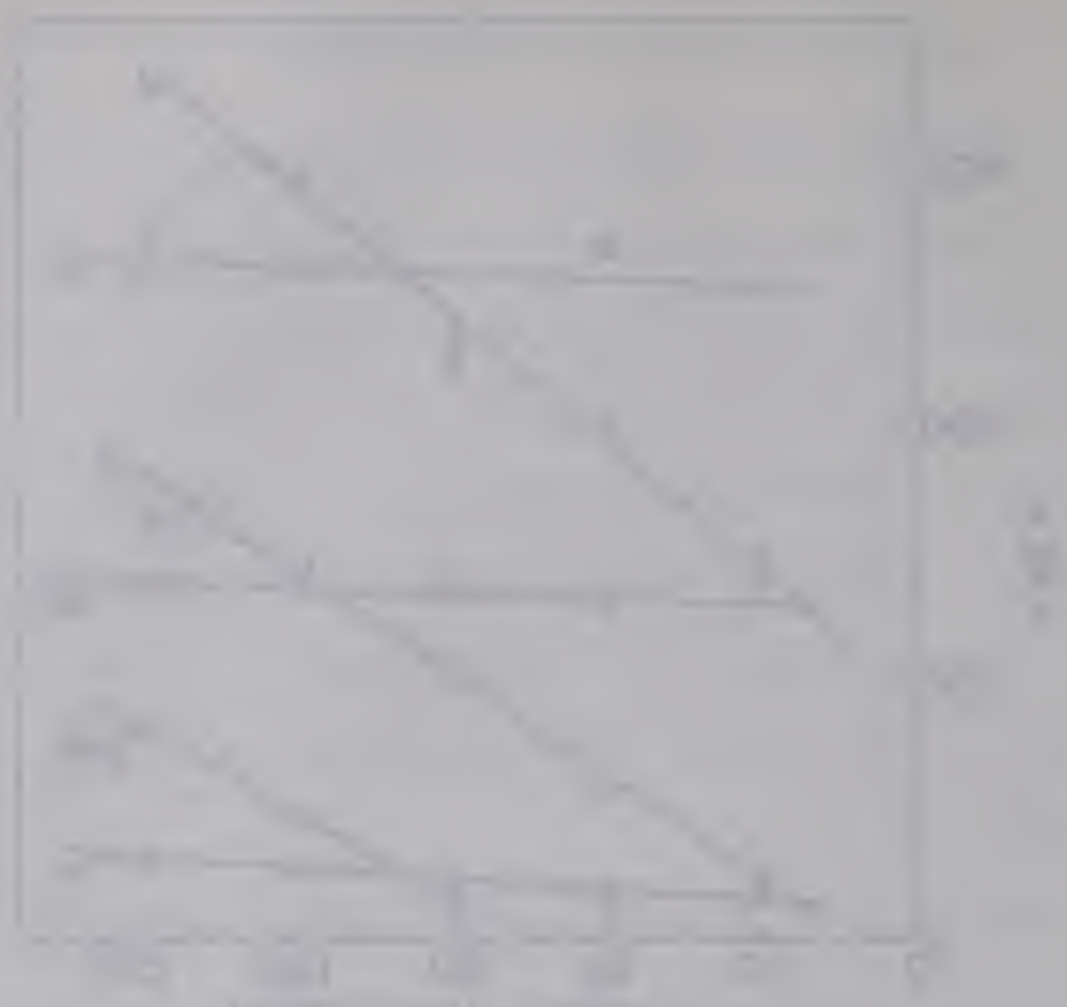


Figure 12: Reaction of phosphorylase a peptides with iodoacetamide. 1.62×10^{-5} M phosphorylase a was reacted with 10 mM iodoacetamide in 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5. The peptides were separated as described in fig. 9 and the area under the peaks on the strip scanner determined using a planimeter. The areas shown are direct planimeter readings.



are involved in the reaction, then fig. 12 is best interpreted as follows:

i) B1, B2, and C are variants of the same peptide which react rapidly with iodoacetamide, but which are not concerned with the inactivation of the enzyme by this reagent.

ii) The N peptide and the A peptide are two distinguishable peptides (already proven by the sequence determination), both concerned with the inactivation reaction, but having different reaction rates.

iii) The E and D peptides are variants of either the A or N peptides or of both of them.

The areas shown in fig. 12 and the areas from a similar experiment for phosphorylase b were used to calculate reaction rates for the peptides. The total area of the peaks at each time was set equal to the total cpm incorporated at that time, and from the area of each peptide as a percentage of the total area, the cpm incorporated into each peptide at each time were determined. The percent reaction of each peptide at each time could then be calculated. A plot of

$$\log \frac{100\%}{\%\ \text{unreacted}}$$

for the reaction of phosphorylase a is shown in fig. 13 and the pseudo-first order plot for phosphorylase b is shown in fig. 14. The rate constants from both figures are shown in Table III, along with a rate calculated from fig. 16 for the sum of the B peptides.

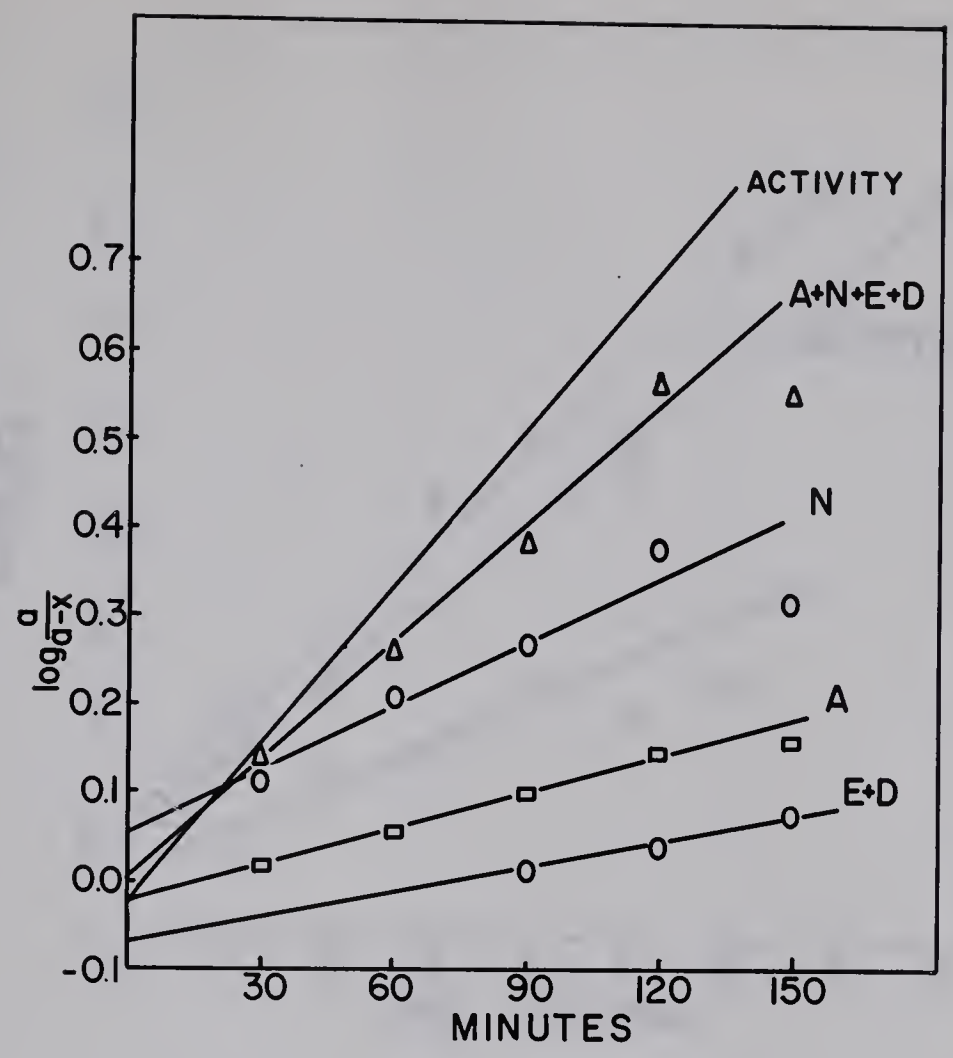


Figure 13: Kinetics of incorporation of ^{14}C-iodoacetamide into phosphorylase a peptides. Log $\frac{100}{\text{fraction unreacted}}$ for the reaction of each peptide was calculated as described in the text. The values for each peptide at each time were summed to give the values for the line A + N + E + D. Activity was determined for the same reaction mix at the times specified, and the values obtained subjected to computer determination of a least squares fit, giving the line shown.

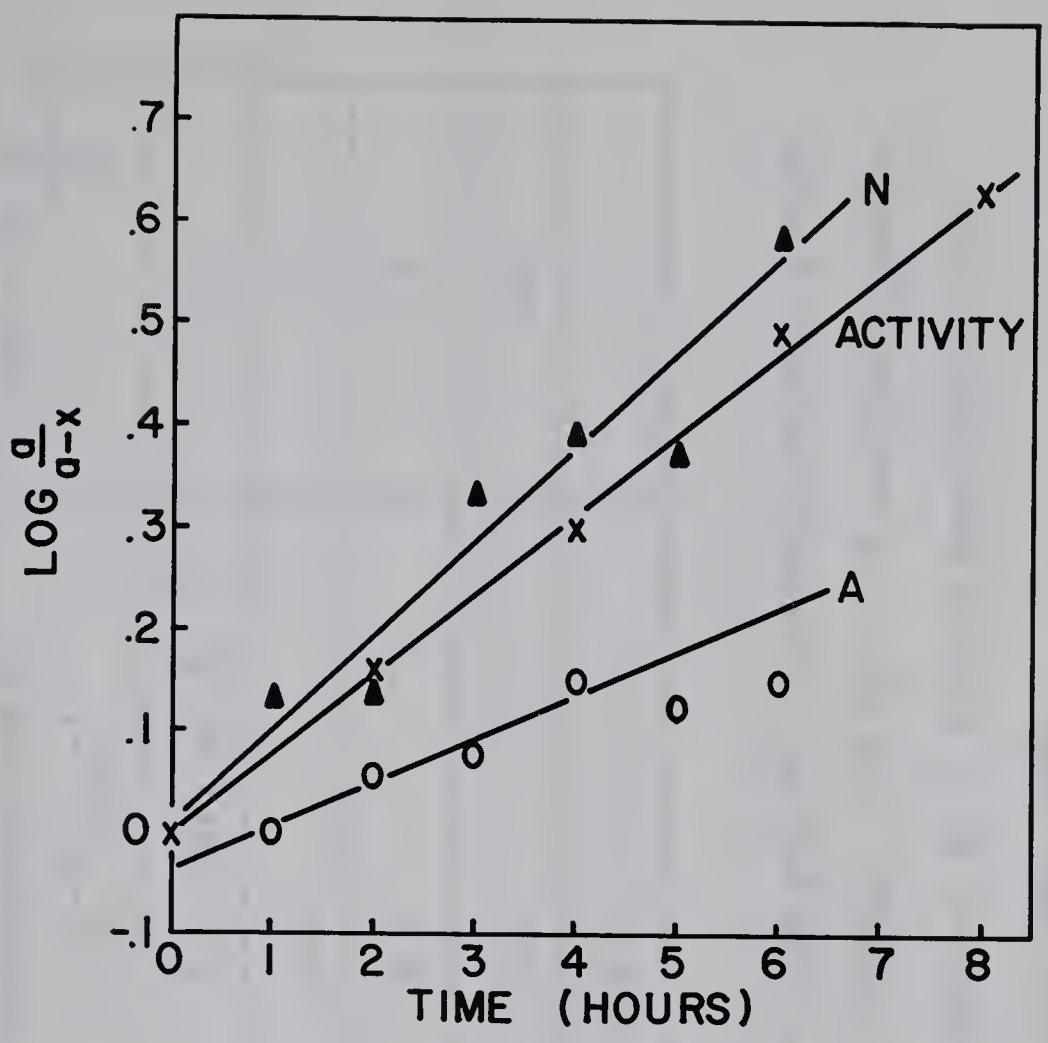


Figure 14: Kinetics of incorporation of ^{14}C-iodoacetamide into phosphorylase b peptides. Log $\frac{100}{\text{fraction unreacted}}$ for each peptide was calculated as described in the text. Activity determinations were done as previously described.

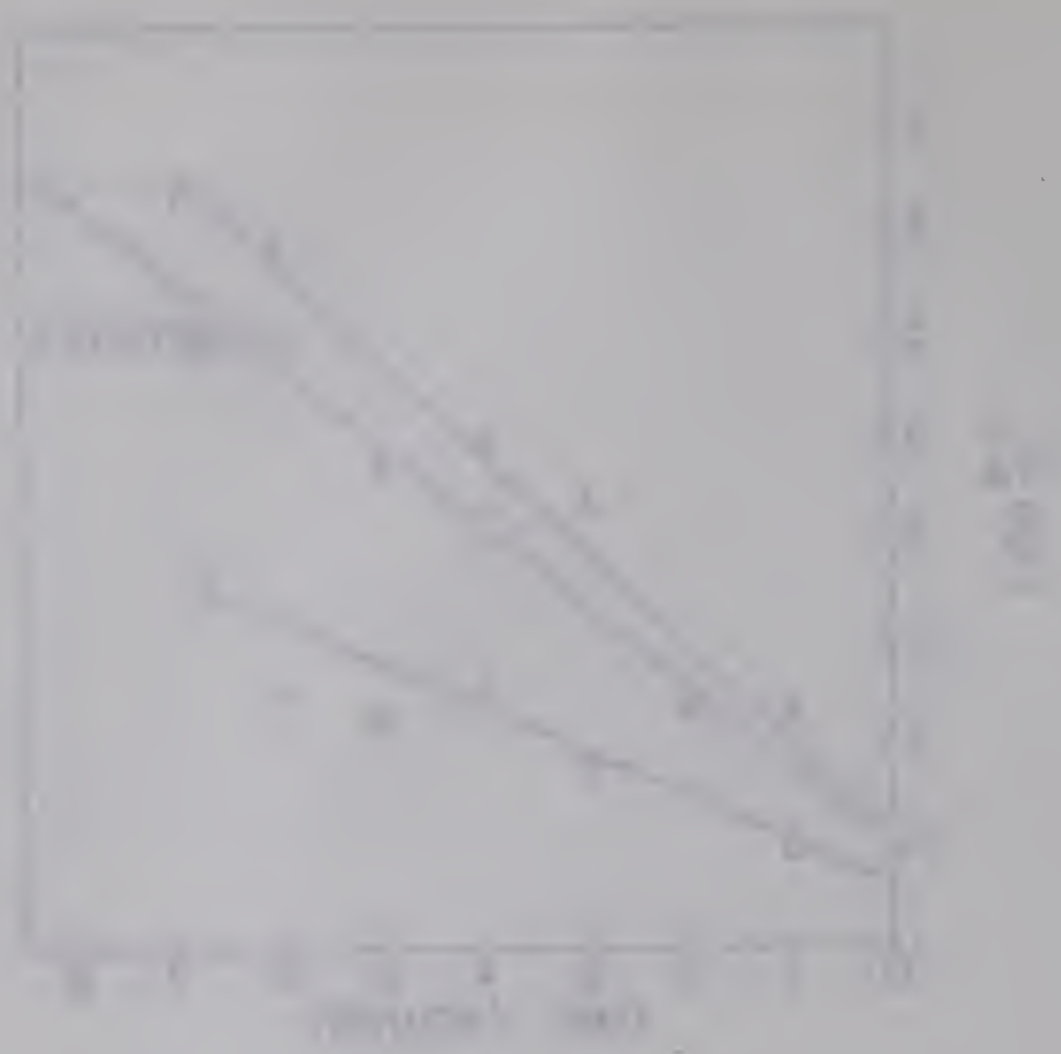


TABLE III: Reaction of Phosphorylases with Iodoacetamide;

Pseudo-first order rate constants

	Phosphorylase a x 10^{-3} min^{-1}	Phosphorylase b x 10^{-3} min^{-1}	Ratio of $\frac{a}{b}$
Inhibition	13.5	3.4 (av. of 4)	3.8
A + N*	10.7	5.3	2.0
N	5.4	3.6	1.5
A	3.1	1.7	1.8
B1 + B2		145	

Reaction of 9 mg per ml of phosphorylase b or 6 mg per ml of phosphorylase a with 10 mM iodoacetamide as described previously. Buffer: 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5 at 30°. The rate of B1 + B2 was measured in an experiment where the iodoacetamide concentration was 1 mM.

*plus the variants which appear with time.

5) The B peptide

Further evidence to support the conclusion that B1 and B2 are variants of the same peptide rather than two unique peptides is shown in fig. 15. The top line shows the total incorporation of ^{14}C-iodoacetamide while the bottom line shows the reaction of the sum of B1 and B2 calculated as described in the previous section. When the lower line is extrapolated to zero time, a value of 78% is obtained indicating that 78% of B1 + B2 have reacted very rapidly. When the value for the total is extrapolated to zero time, 71% of all the groups are slowly reacting. That is, 29%, equal to 78% of 1/3 of the total groups, have reacted rapidly. The conclusion is that B1 and B2 are variants of the same peptide.

Another experiment was done in which the conditions were adjusted to give a much slower reaction. (Conditions specified under fig. 16). The following table shows the activity as units per mg of protein compared to the incorporation of iodoacetamide per mole of dimer.

TIME	ACTIVITY	MOLES INCORPORATED
30 sec.	1860	0.10
5 min.	2020	0.84
20 min.	2240	1.52
40 min.	2090	1.61
60 min.	2200	1.60
80 min.		1.60
100 min.	2080	1.68
120 min.	2130	1.58

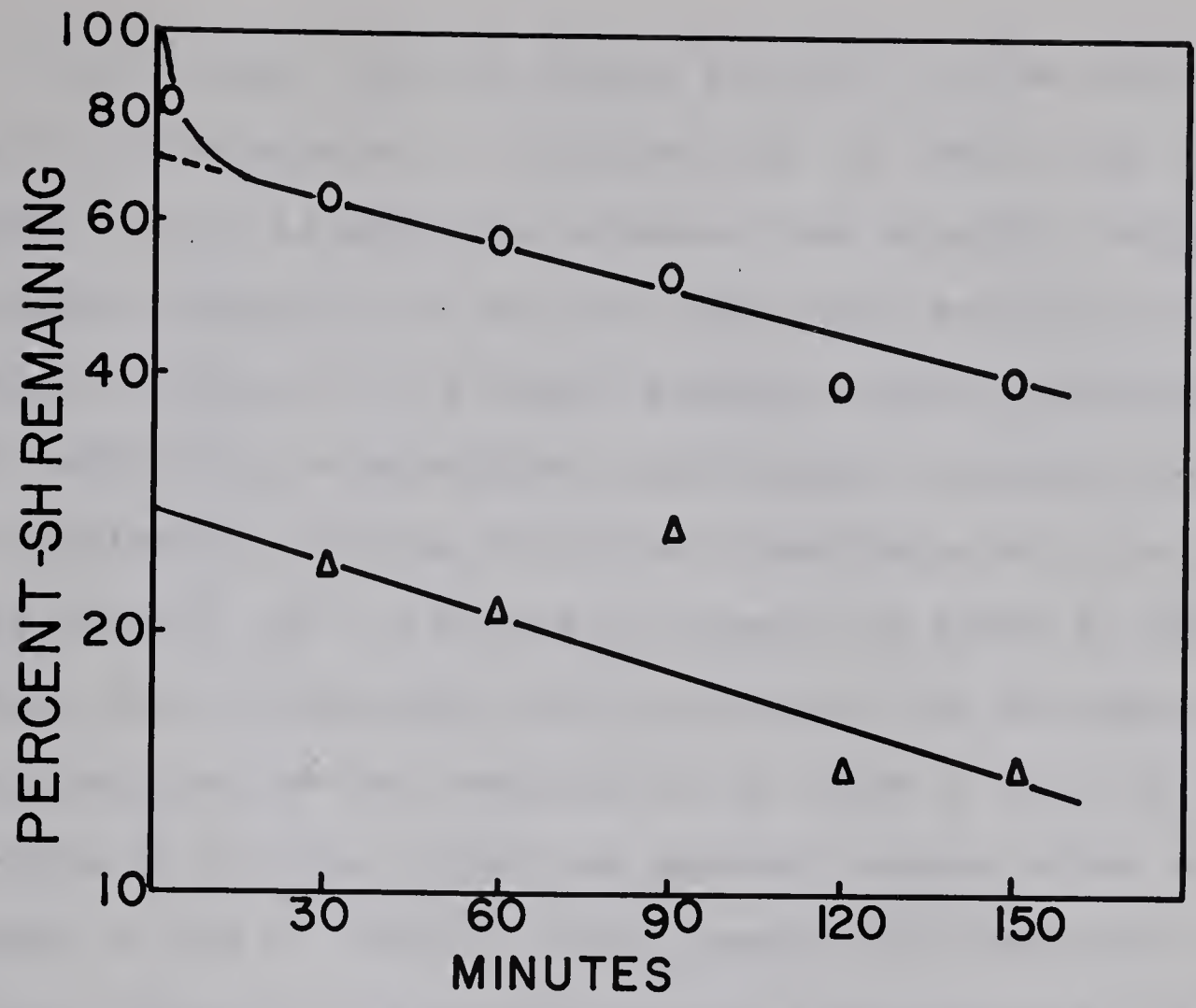


Figure 15: Total incorporation of ^{14}C-iodoacetamide compared to reaction of B1 and B2 peptides of phosphorylase a. The percent of reaction of each peptide was calculated as described in the text.

O -- total incorporation as a percentage of 12 -SH groups per mole of phosphorylase a.

Δ -- sum of reaction of B1 and B2 subtracted from 100.

It is evident that no enzyme activity is lost while 1.6 moles of iodoacetamide is incorporated, or 80% of one -SH per monomer. There is previous evidence that specific -SH groups in certain proteins (36) are not completely available for reaction. Further it is highly possible that a percentage of the -SH group is disulfide interchanged in native protein. (The possibility of some disulfide interchange will be discussed later.) If 1.6 groups per dimer are taken as 100% reaction then a half-time for the reaction can be taken from a semi-log plot of the reaction as is shown in fig. 16. The half-time is 4.6 min. giving an apparent second order rate constant of 144 M^{-1} min^{-1}. This constant is comparable to the rate constant of glutathione with iodoacetamide (75 M^{-1} min^{-1} at pH 7.5, calculated from the work of Guidotti, 37; see also 38, 39). This rate is much faster than the reaction rate for any of the other -SH groups of phosphorylase. The N peptide -SH group of phosphorylase a has an apparent rate constant of 0.54 M^{-1} min^{-1}, while those of the other -SH groups are even smaller.

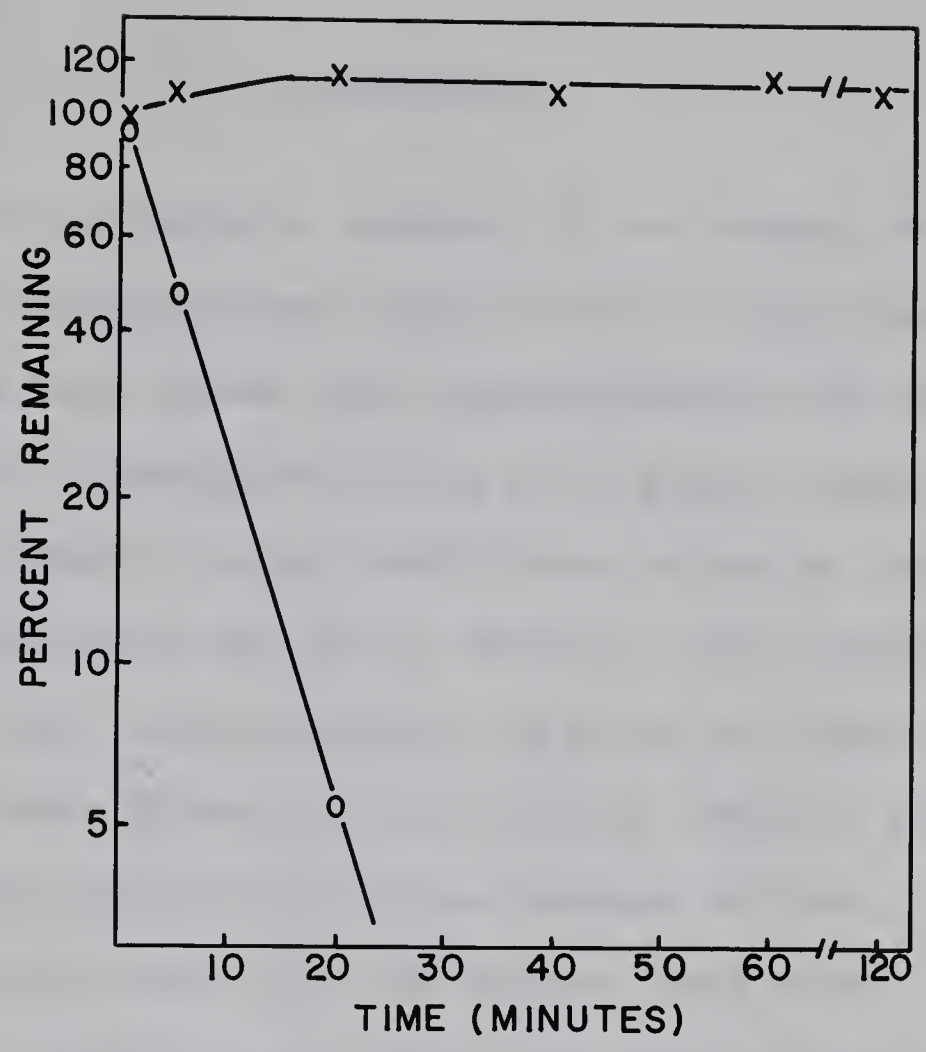


Figure 16: Reaction of B1, B2 peptide of phosphorylase b with ^{14}C-iodoacetamide. Reaction of 1.05 x 10^{-3} M iodoacetamide with 6.00 x 10^{-5} M phosphorylase b in the presence of 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, 0.04 x 10^{-3} M mercaptoethanol, pH 7.5. Incorporation plotted as a percentage of 1.6 -SH groups per mole of phosphorylase b remaining. Activity determined as described in fig. 5.
X -- activity as percentage of zero time value remaining.
O -- incorporation as percentage of 1.6 -SH groups.

DISCUSSION

Table IV contains a summary of the number of -SH groups per mole of phosphorylase dimer reacting with the reagents studied. We have shown that approximately two -SH groups per molecule of phosphorylase b will react rapidly with a variety of reagents under conditions in which the enzyme remains undenatured and fully active. The reagents include iodoacetic acid, iodoacetamide, cystine and DTNB. Jokay et al. (40) have shown that the latter reagent reacts with two -SH groups while the enzyme remains active. It is noteworthy that these two -SH groups react with iodoacetamide at least as rapidly as do model compounds, and it must be concluded that they are fully exposed on the enzyme surface. It would appear that a mixed disulfide can be formed with this one particular -SH per mole of phosphorylase monomer without the loss of activity, and it is quite possible that such compounds may occur naturally under in vivo conditions, or that inter-molecular disulfides might connect the enzyme molecules into larger aggregates, such as are sometimes seen in the ultracentrifuge (10). It should be noted that the aggregates seen are usually found to have sedimentation constants in agreement with those expected for distinct combinations of phosphorylase. The possible role of these highly reactive -SH groups in modulating the regulation of activity of this complicated allosteric enzyme remains to be

TABLE IV: Summary of Reaction of Phosphorylase with -SH Reagents

Reagent	Denaturing Agent	-SH groups reacting / dimer
A. Conversion to cysteic acid		18
B. PCMB	None	6-7
	SDS	11
	Acid	12
C. DTNB	None	1-3
	SDS	11
	Urea	9.5
	Guanidine HCl	9
D. IAA	Urea	12
E. IAM	None (No inactivation)	1.6
	None (Inactivated)	6-7

Note: The values stated are correct (per dimer) for either phosphorylase a or phosphorylase b except for the last value stated for IAM, which will be discussed later.

investigated, but the formation of similar mixed disulfides with fructose diphosphatase has been shown to affect the activity of that enzyme (41).

It has also been shown that PCMB and iodoacetamide will react with a second class of four -SH groups per molecule of native phosphorylase b, and that the titration of these groups results in the complete loss of activity, as well as the dissociation of the protein into monomers. These -SH groups react much more slowly than the first two, or than model compounds, and they must therefore be "masked" in some manner. The results suggest that there must be two specific -SH groups on each monomer unit which, when titrated, are responsible for the loss of activity and the structural changes. The two cysteinyl peptides which these kinetic studies have predicted have been isolated and identified.

The isolation of the -SH group containing peptides has now nearly been completed. The efforts of Mr. C. Zarkadas (42) made it possible to identify the peptides containing the reactive -SH groups from amino acid analysis and a single N terminal determination. The problems encountered in the course of Mr. Zarkadas' work illustrate the difficulty of applying methods developed for use on small proteins to a protein the size of phosphorylase. Pepsin is the only enzyme which splits the enzyme sufficiently to allow for isolation of relatively low molecular weight peptides but it is so non-specific that more than one form of each peptide is isolated

in many cases, making it necessary to sequence the same peptide several times in the course of the work. In this respect, it can be added that the two forms of the basic peptide (B1 and B2) appear to occur in the same ratio in each digestion mix, when the protein had been subjected to similar treatment prior to digestion.

The work of Madsen and Cori (10 - see fig. 1) indicated that each of the -SH groups of phosphorylase a which were available for reaction at neutral pH, was equally necessary for the activity of the enzyme. Our work has shown that one of the three -SH groups per monomer which react at neutral pH, the B1, B2 -SH group, is not necessary for activity. It is possible that the result obtained by Madsen and Cori was due to the procedure for isolating the enzyme. Cysteine was used throughout the procedure to protect the -SH groups, whereas in the work reported here, mercaptoethanol was used. Since the formation of mixed disulfides with other proteins has now been shown to occur both in vitro (43, 44) and in vivo (45), and since we have shown that the -SH group in phosphorylase can react readily with cystine, it can be postulated that the particular -SH group might be interchanged in vivo, and thus would have been unavailable for reaction with PCMB. Fig. 17 shows a plot of activity of phosphorylase b against the number of moles of -SH reacted per dimer with iodoacetamide. In the two experiments shown, two -SH groups per dimer can clearly be reacted without loss of activity. This result was not

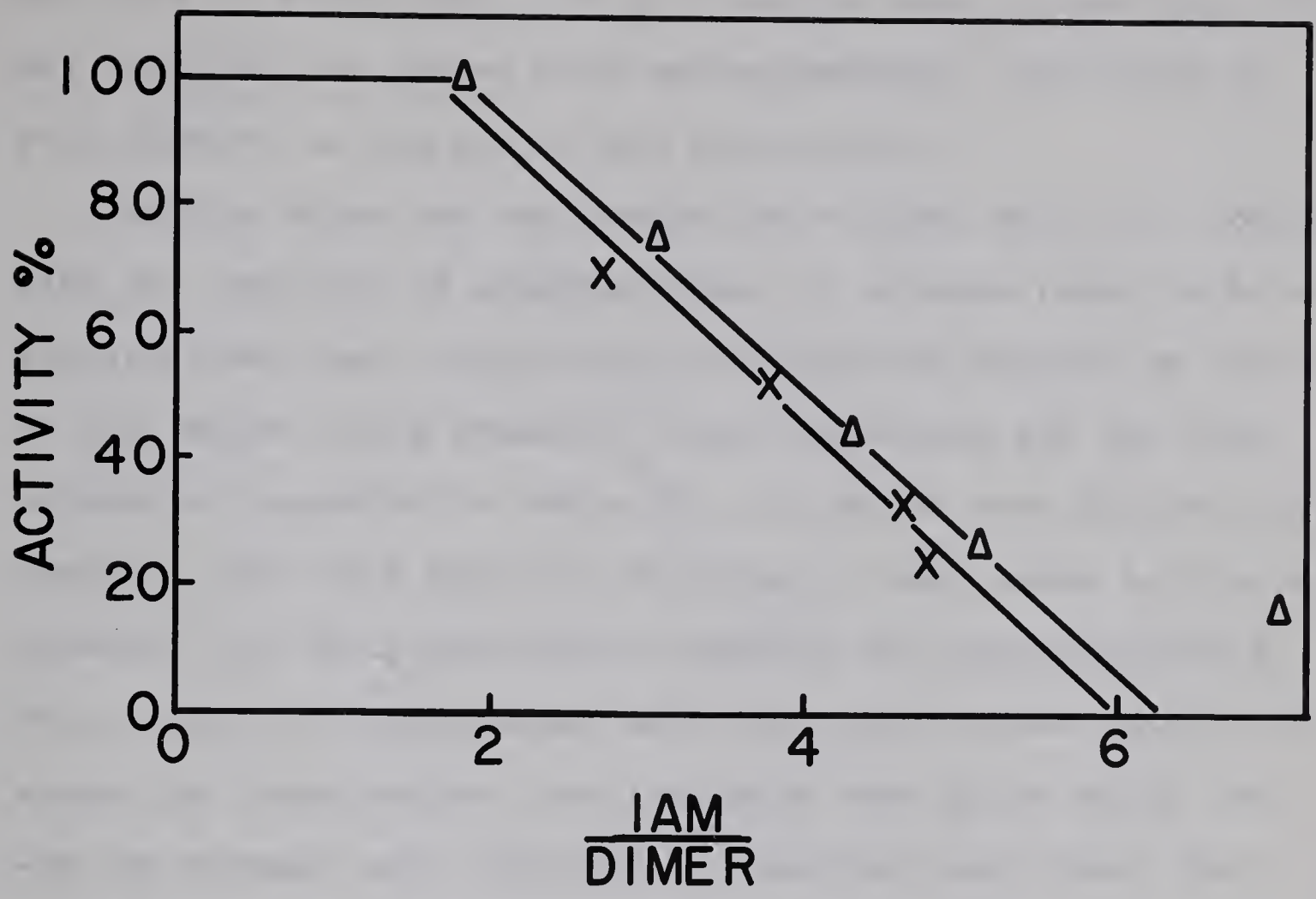
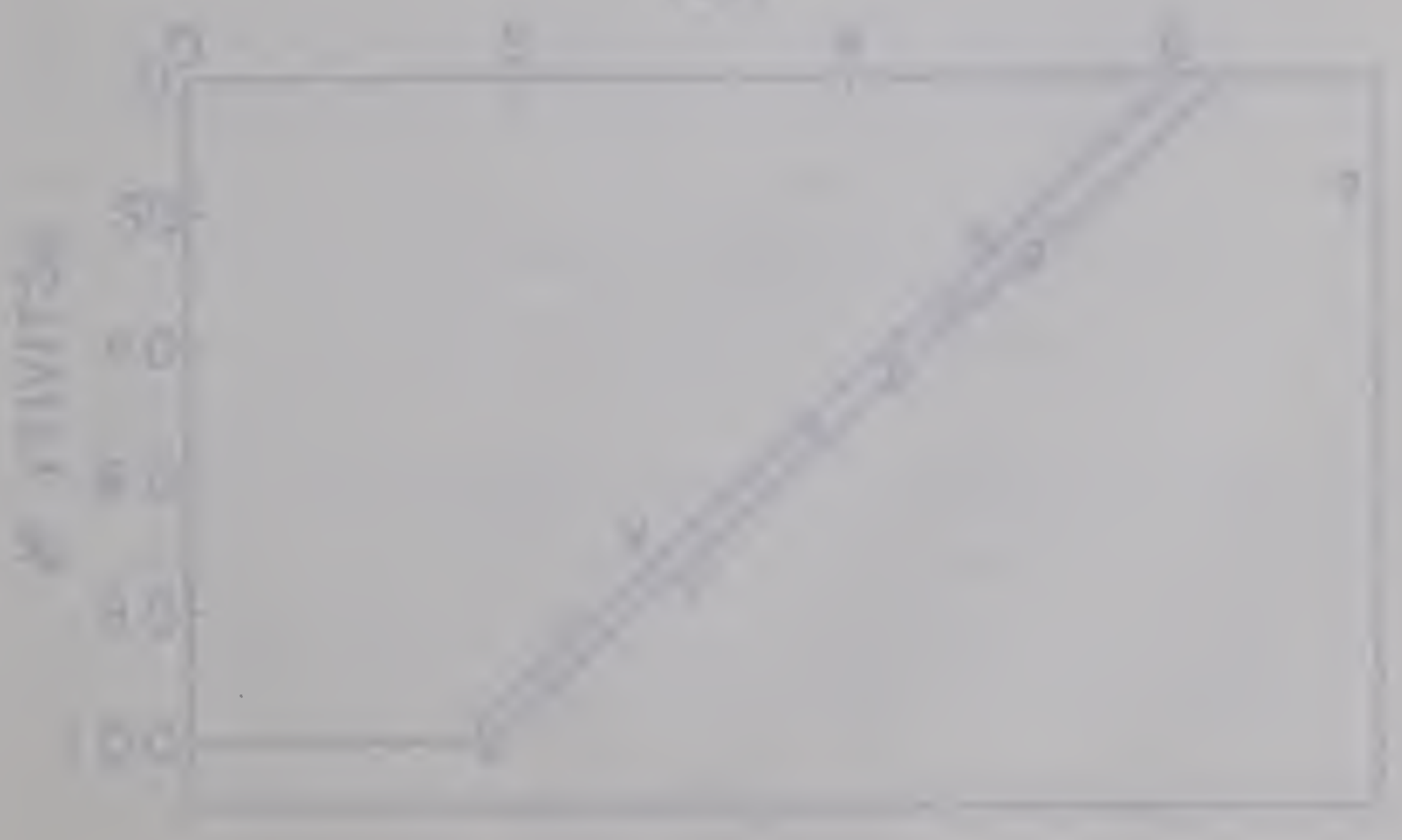


Figure 17: Effect of reaction of phosphorylase b -SH groups with ^{14}C-iodoacetamide on activity of the enzyme. Values taken from two experiments where incorporation of iodoacetamide was measured along with inactivation of the enzyme. Reaction conditions in both cases: 10 mg per ml phosphorylase b, 10 mM iodoacetamide, 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5.



obtained in every case. It is possible that a mixed disulfide may sometimes be formed with mercaptoethanol, depending on such factors as the age of the preparation.

Of the other two -SH groups per monomer which are concerned with the activity of phosphorylase, it appears from the kinetic studies that their relationship to activity depends on the form of the enzyme being studied. From the values for the rate constants presented in Table III, it can be seen that the rate constant for the N peptide -SH group is very close to the rate constant for the inactivation reaction for phosphorylase b. This is not in disagreement with the result shown in Fig. 17 since the inactivation reaction could take place while two -SH per monomer were reacting, giving the line shown, but could depend on the reaction of only one group.

In contrast, the results for phosphorylase a suggest that the sum of the reaction rates for the A and N peptides is close to the overall reaction rate (see Table III). An analysis of the data according to the method of Ray and Koshland (46, 47) suggests the reaction of either one or the other -SH group results in an inactivated form of the enzyme. The curve for this reaction shown in Fig. 18, indicates also that the reaction of either group tends to make the other group unreactive (perhaps inaccessible) so that the curve extrapolates to a total of two -SH groups per monomer reacting with the accompanying loss of 100% of the activity.

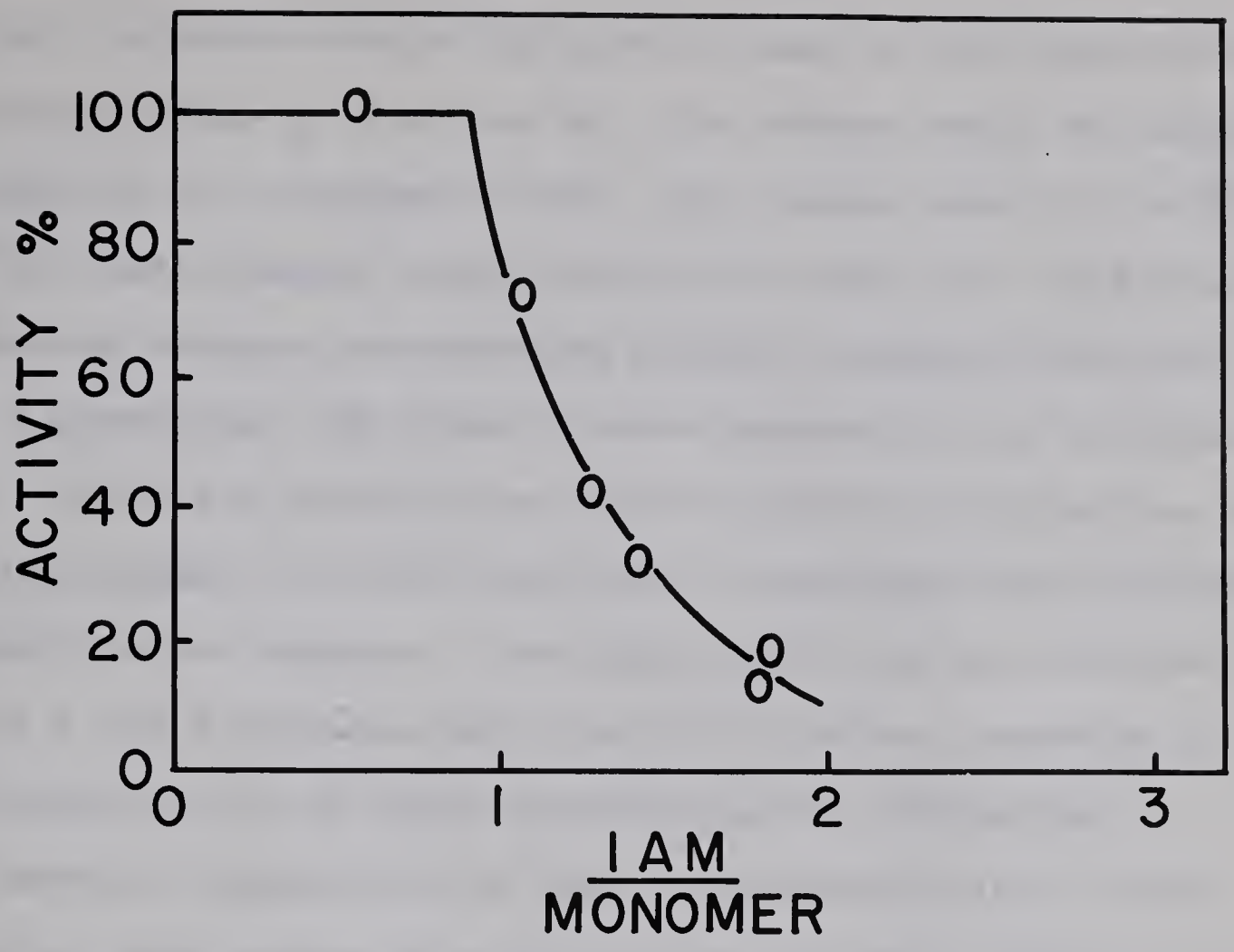
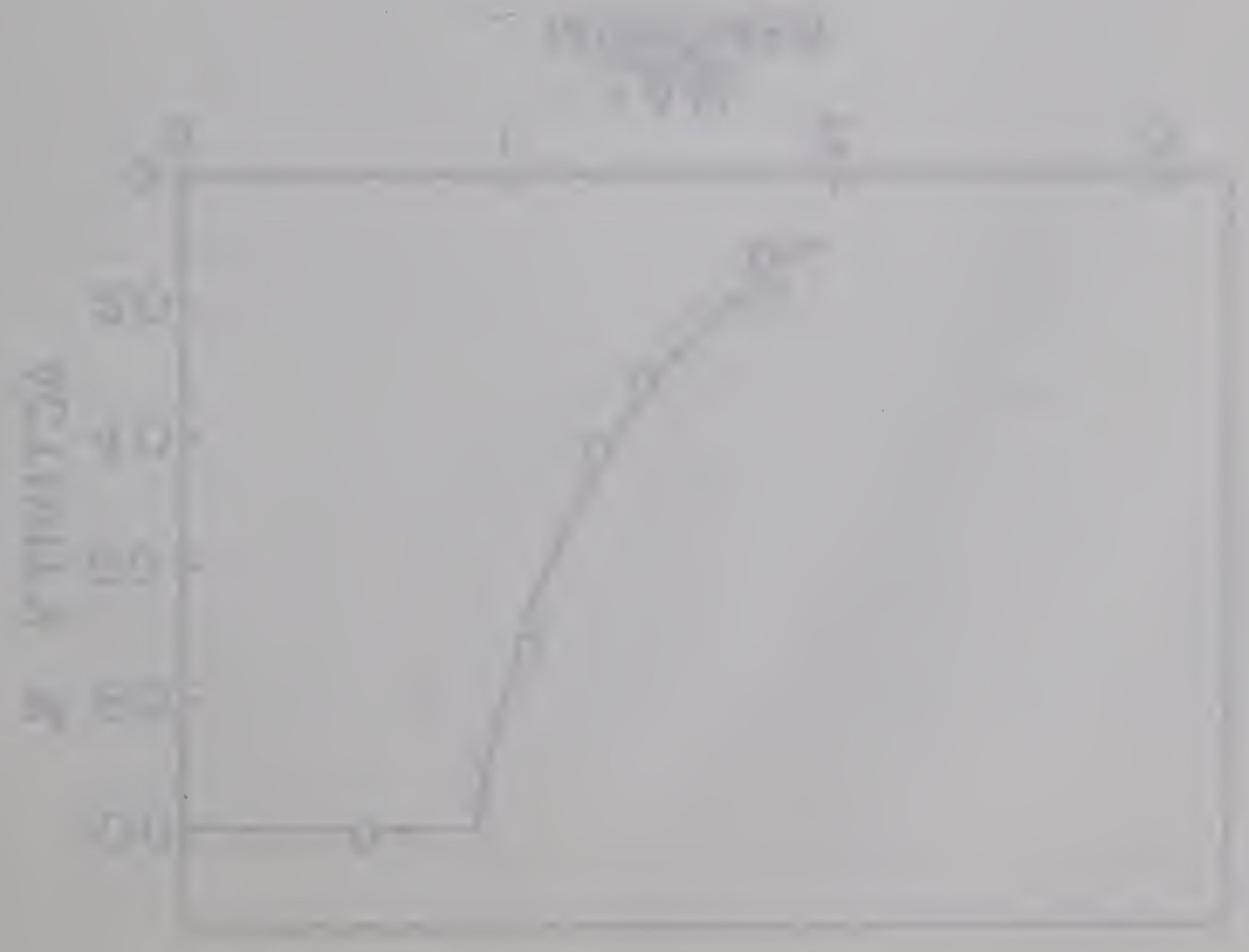


Figure 18: Effect of reaction of phosphorylase a -SH groups with ^{14}C-iodoacetamide on activity of the enzyme. 6.0 mg per ml phosphorylase a in 1% KCl, 1% sodium glycerophosphate, 1.5 mM EDTA, pH 7.5 was treated with 10 mM iodoacetamide.



At the concentration of protein used in the experiments on phosphorylase a (6 mg per ml), the enzyme would be expected to exist in its tetrameric form. The faster rate of inactivation by iodoacetamide might therefore be due to a conformational difference between tetrameric and dimeric phosphorylase such that a particular -SH group is more exposed in the tetramic form. Since the differences in the allosteric properties of the two enzymes are often ascribed to conformational differences between the two enzymes, investigation of the reactivities of the A and N peptides may provide a chemical approach to the investigation of these conformational differences.

Another explanation is that the phosphorylated serine group may make attack on a particular -SH group easier. It should be noted that the hexapeptide which surrounds the phosphorylated serine residue (7) does not contain a -SH group. However, the -SH group might be in close proximity in the three-dimensional structure of the enzyme.

The remaining two-thirds of the -SH groups are so inaccessible, so well protected by the tertiary structure of the protein, that they can usually not be titrated with any reagent until the protein is thoroughly denatured. The methods of denaturation which we have used, including 8 M urea, 5.2 M guanidine HCl, 1% SDS and pH 2.3, are classically considered to completely denature proteins and to fully expose all of the -SH groups. Thus, any of the reagents which have been shown to react with normally exposed -SH groups

would be expected to titrate all of the -SH groups in phosphorylase subjected to the above denaturing conditions. This is not the case. There is variation of reactivity for the same denaturing agent as shown by the fact that cystine reacts with only 6 -SH groups per mole of phosphorylase b in 8 M urea, while DTNB reacts with 10 and NEM and iodoacetic acid reacts with 12 to 14. There is also considerable variation in the reaction of the same reagent in different denaturing conditions, e.g., DTNB reacts with 12 -SH groups per mole of phosphorylase b in 1% SDS, with 10 in 8 M urea, and with 9 in 5.2 M guanidine HCl. None of the above reagents in any of the denaturing conditions were able to titrate all of the -SH groups in phosphorylase and this work points out the caution which must be exercised in interpreting such experiments.

The apparent non-reactivity of varying numbers of -SH groups in phosphorylase under denaturing conditions is difficult to interpret. We hope that one possible explanation, the oxidation of the -SH groups to disulfides upon denaturation, has been ruled out by the titration at pH 2.3, or at neutral pH in the presence of protective agents. It is possible that a certain degree of steric hindrance is still present in phosphorylase after denaturation.

While it is difficult to eliminate unambiguously all possibility of some disulfide bridges in native phosphorylase, our results do not provide any support for this idea. It

should be noted that at least four of the half-cystine residues in phosphorylase b would have to be unaccounted for as -SH groups in order to permit one disulfide bond per monomer.

Some investigators have reported results that are at variance with ours. For example, Damjanovich and Kleppe (48) reported that they could react nearly all of the -SH groups in phosphorylase b with DTNB in 0.9% SDS, whereas we can react only about two-thirds of them under these conditions. However, a calculation of the extinction coefficient for the colored anion of DTNB which was employed by these workers revealed that they had obtained a value of 9,500. This is much lower than Ellman's value of 13,600 (24), and we have confirmed the latter. If the results of Damjanovich and Kleppe are re-calculated for the correct extinction coefficient, and corrected for the new molecular weight, then they agree with the values reported in this paper. They also obtained higher values when using PCMB in 0.9% SDS, but this may have been due to preparing their protein solution in 0.033 M glycerophosphate, pH 6.8, containing 0.0001 M EDTA. The chelating agent increases the absorbance of PCMB and therefore interferes with the spectrophotometric titration of -SH groups (23).

We have, for convenience, divided the -SH groups of phosphorylase into four classes on the basis of their reactivity. However, the number of -SH groups which react under any particular set of circumstances is determined by the

ability of each of the nine differently positioned groups (per monomer) to react with the particular reagent chosen. There appears to be a gradation of reactivity such that, if conditions could be found in which every -SH group would react with a particular reagent, a different rate constant would be found for each -SH group.

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